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A Novel Approach to Managing Invasive Termite Species Using Genetically Engineered Bacteria

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14. ABSTRACT

The hind gut of the Formosan subterranean termite (FST) is a refuge for an array of protozoa and bacteria that fulfill important functions in the survival of their hosts. Due to their importance for FST survival these symbionts are excellent tools or targets for the control of FST. Lytic peptides were tested as protozoicides. Lytic peptides were shown to kill the termite symbiotic protozoa and this defaunation was associated with the subsequent death of termites. A prototype delivery system was developed using the yeast Kluyveromyces lactis and the lytic peptides Hecate and Melittin. Studies showed defaunation of termite workers when fed with lytic peptide expressing yeast. Conditions to genetically engineer termite-specific bacteria were investigated and the enzyme profile of the termite gut was determined. These studies are crucial for development of a bacterial shuttle to produce toxins that are activated by enzymatic cleavage in the termite gut and with further development, the system could be an environmentally friendly, cost effective alternative to chemical baiting systems presently used to control termites

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Acronyms and Abbreviations

dH_2O	Deionized water
EK	Enterokinase
ELISA	Enzyme-Linked Immunosorbent Assay
GFP	Green Fluorescent Protein
MBP	Maltose Binding Protein
NEB	New England BioLabs
PCR	Polymerase chain reaction

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Mention of names of supply companies and product names does not imply endorsement of either the company or the product.

Executive Summary

Justification of the project: Subterranean termites are one of the most destructive and costly pest species in the United States and in many other countries. These wood-destroying insects are a major concern for the Department of Defense because it has billions of dollars invested in wooden structures and products. Termite management costs are second only to those for cockroach control. Invasive termites are easily transported around the world with movement of wooden material. Therefore, there is increasing demand for the development of new environmentally friendly, fast, and cost-effective termite control technologies, preferably without the use of chemical pesticides.

Paratransgenesis for termite control: We proposed to develop a novel approach to termite control employing paratransgenesis, i.e. the use of genetically engineered symbiotic microorganisms as "Trojan Horses" to deliver and spread detrimental gene products in a termite colony. We proposed to genetically engineer indigenous gut bacteria isolated from the invasive Formosan subterranean termite, *Coptotermes formosanus* Shiraki to express lytic peptides in the termite gut. Lytic peptides were expected to destroy the symbiotic protozoa in the termite gut, which are responsible for the majority of cellulose digestion, and thus cause death of the termites through starvation. The bacterium would be expected to spread through the colony via social interactions between the colony members, such as grooming and trophallaxis. Since the natural habitat of the bacterium is the termite hindgut, the control agent would be naturally self-replicated in the gut. Thus, a limited amount of product would be needed to eradicate a termite colony. Since termite gut microorganisms have limited survival capability outside of the gut and microorganisms are not exchanged between colonies, this control method would be confined to eradicate single colonies of termites and thus minimize negative impact on non-target organisms and the environment. Paratransgenesis would provide a highly specific approach to control insects in urban and agricultural environments without the use of conventional pesticides. Bait systems could be used to introduce a live biological control agent that produces lytic peptides to kill the obligatory symbionts of target pests, such as termites and cockroaches.

Previous studies proved the principle of paratransgenesis: The bacterium *Enterobacter cloacae* was isolated from the gut of the Formosan subterranean termite and genetically engineered to express green fluorescent protein (GFP). This bacterium was able to deliver and express foreign genes in the termite gut and was rapidly transferred among workers and soldiers in laboratory colonies (Husseneder et al. 2005a; Husseneder and Grace 2005). However, the GFP bacteria were only used as a monitoring system to confirm uptake, spread and long-term survival of genetically modified bacteria in a termite colony; no detrimental gene products were expressed for termite control. Preliminary data suggested that lytic peptides kill the termite symbiotic protozoa, which are required for the termite host's survival, *in vitro* (Hierath et al. 2002). Therefore, lytic peptides were the detrimental gene product of choice for the development of a paratransgenesis system for termite control.

Goals and milestones of this project:

a) Proof of concept. The goal of this project was to provide proof of concept that a microbial shuttle system could express protozoacidal gene products in the termite gut that would defaunate and kill termites. Furthermore, several methods to increase environmental safety were

explored to optimize the paratransgenic system before application for termite control could be considered. The initial phase of this study involved the development of methods to test the efficacy of lytic peptides against protozoa. Aerobic cultures of the free-living protozoa, *Tetrahymena pyriformis*, were used to test lytic peptide *in vitro*. The three species of protozoa from the termite gut were cultured *in vitro* using anaerobic culture methods outlined by Trager (1934) and used to test lytic peptide activity anaerobically. The lytic peptides killed protozoa under both aerobic and anaerobic conditions. A microinjection system was then developed and optimized to test lytic peptide activity *in vivo*. Small volumes (~0.5 µl) of different lytic peptides were injected into the hindgut of termite workers. Following injection of the lytic peptides Hecate and Melittin, defaunation of injected termite workers was observed at 72 hrs. A separate experiment in which termite workers were fed the protozoacidal drug Metronidazole (Flagyl), confirmed that defaunation led to termite death after six weeks. The lytic peptides Hecate and Melittin were subsequently used to construct a prototype paratransgenesis system for proof of concept that paratransgenesis can be used to kill termites.

The host selected as the paratransgenesis prototype was the commercially available yeast, *Kluyveromyces lactis*. Ingestion of yeast by termite workers was shown using fluorescence-labeled yeast that could be detected in worker's hindguts by fluorescence microscopy. The genes for expression of Melittin and Hecate, i.e. the lytic peptides that had been determined to efficiently kill protozoa *in vitro* and *in vivo*, were codon optimized for expression from yeast and commercially synthesized. The gene sequences were cloned into *K. lactis* and expression of active lytic peptide was confirmed by observing death of *T. pyriformis* cultures when they were treated with yeast supernatant. When termite workers were fed with the lytic peptide expressing yeast, their guts were defaunated within 4 weeks. The yeast-based prototype paratransgenesis system provided proof of concept that a symbiotic microorganism can act as a "Trojan Horse" and express protozoacidal peptides to defaunate the termite gut. As shown with the protozoacidal drug Metronidazole, defaunation leads to termite death. Thus proof of concept that paratransgenesis can be used to kill termites was achieved.

The "killer yeast strains" lost their capability to express lytic peptides two months after initial confirmation of their toxicity due to recombination events at the genomic level. While loss of lytic peptide expression over time increases environmental safety, further investigation into long-term storage of yeast stocks (e.g. lyophilization) will be required before a yeast-based bait system can be developed.

b) Measures to increase environmental safety: Several additional safety features that would reduce non-target effects of the paratransgenic system were explored. These features included using termite-specific bacteria as microbial hosts instead of yeast, activating lytic peptides through cleavage by termite-specific gut enzymes and the use of ligand-linked lytic peptides to target surface receptors of the protozoa.

Termite specific bacteria were previously identified using culture independent 16S rRNA gene sequencing of the gut content of Formosan termite workers (Husseneder et al. 2005b). Bacteroidetes was the dominant phylum and was found in all termite colonies, including colonies from Japan (Shinzato et al. 2005; Noda et al. 2005). *Dysgonomonas* spp. (order Bacteroidales) were previously cultured (Husseneder et al. 2005b) and suggested as potential candidates for termite-specific microbial hosts. However, the gram positive lactic acid bacterium *Pilibacter termitis*, which is the dominant species in culture and is present in most termite colonies across all investigated geographical regions (Husseneder et al. 2005b), was selected as the primary

microbial host, because gram positive bacteria are less susceptible to lytic peptides than gram negative bacteria, such as the *Dysgonomonas* spp.

Conditions for the genetic manipulation of the novel, indigenous termite gut bacteria had to be determined. Previous literature suggested that transformation was most likely to succeed if attempted in the mid-late exponential growth phase. Therefore, growth curves for *P. termitis* were created and the mid-late exponential phase determined to be ~18 hrs post inoculation. Transformations using chemical methods to disrupt the cell wall and electroporation techniques employing numerous species of plasmids; electroporation buffers; voltage settings and bacterial recovery time were investigated. So far, none of the cell wall disrupting enzymes such as Mutanolysin and Lysozyme and none of the attempted combinations of electroporation conditions yielded successful transformation.

Construction of a paratransgenic system that secretes an inactive pro-toxin that is specifically cleaved and activated by termite-specific enzymes once it enters the gut environment would ensure an environmentally safe system of termite control. In search of such termite-specific gut enzymes, the general enzyme profile of the termite gut was established and metallo-proteases and serine proteases were identified. To date, 23 of the unknown proteins have been sequenced, but no unique protease was found so far.

Identification of unique receptors on the membranes of the termite protozoa would allow the development of protozoa specific ligands to which the lytic peptides could be fused. Initial investigations focused on the identification of insulin receptors, as insulin-like receptors had been identified in *T. pyriformis*. Protozoa were observed *in vitro* internalizing fluorescently labeled insulin, however when using an ELISA kit designed for insulin receptor identification, no insulin receptors were observed on the surface of the protozoa in the termite gut.

Accomplishments and future research: The funding from the SERDP has ensured that vital milestones have been met and proof of concept was achieved towards the use of a paratransgenic system as a novel approach to termite control. A prototype was constructed by genetically engineering yeast to secrete lytic peptides, which successfully defaunated termites. It was shown that defaunated termites die within a few weeks. With further development, the proposed paratransgenesis system could become an environmentally friendly, cost effective alternative to chemical baiting systems presently used to control termites. The tasks to be completed for the production of a termite specific system include the construction of a termite specific fusion-gene. As envisioned, this fusion gene will express a lytic peptide that is linked to a ligand, which specifically targets surface receptor of symbiotic protozoa in the termite gut. Furthermore, the lytic peptide is expressed in an inactive form and is activated by cleavage by termite specific gut enzymes. The identification of membrane receptors of the protozoa and of unique proteases in the termite gut has not been achieved during the funding period.

As soon as these safety steps are completed a yeast-based system could be commercially developed. Alternatively, transformations of bacteria other than Pilibacter and Dysgonomonas could be attempted. We have isolated over 25 different bacteria species from the termite gut ranging from Enterobacteriaceae that are easily genetically manipulated to other novel species which would require testing different transformation protocols.

1. Objective

The objective of this project was to engineer microbes, which live naturally in the termite gut, to express gene products that are detrimental to termites, a technique known as paratransgenesis. Using paratransgenesis as an alternative to conventional chemical and bait treatments for insect control is an innovative endeavor. To achieve the objective various milestones had to be achieved to establish proof of concept and lay out the path for developing an environmentally- and user-friendly product.

Positive controls, i.e., bioassays with protozoa, had to be developed to test the efficacy of lytic peptides in vivo. A microinjection delivery system had to be optimized to inject lytic peptides into the hindgut of termite workers to establish *in vivo* that termite guts were defaunated by lytic peptides. Also, confirmation that defaunation results in termite death had to be achieved by feeding the protozoicidal drug Metronidazole (Flagyl) to termite workers. Upon determining that lytic peptides defaunate termites and defaunated termites do not survive, a prototype paratransgenesis system was developed, where selected lytic peptide genes were expressed from an easily manipulated yeast expression system. Once tests on the prototype confirmed protozoicidal activity of peptides expressed from the yeast system, the yeast was fed to termite workers and defaunation and mortality was recorded.

Several ways to increase environmental safety were explored. A high level of environmental specificity achieved by using termite specific bacteria as the paratransgenesis host. Other environmental safety measures were to be introduced by engineering a protein-capped lytic peptide fused to a ligand designed to bind to a receptor on the surface of the termite protozoa. This required the identification of receptors on the surface of the termite protozoa and of termite gut specific enzymes, which could not be achieved so far.

2. Background

Termites as invasive pest species: The Formosan subterranean termite (*Coptotermes formosanus* Shiraki) is one of the most destructive and costly invasive pests nationally and internationally. Economic loss due to Formosan termite treatment, damage repair, and demolition of structures ranges in the billions of dollars worldwide. In addition to urban structures and agriculture, this invasive pest species is known to damage military structures and training facilities in temperate, tropical, and subtropical regions. These wood-destroying insects are a major concern for the Department of Defense (DoD) because it has billions of dollars invested in wooden structures and products and termite management costs are second only to those for cockroach control. Invasive termites are easily transported around the world with movement of wooden material, and there is increasing demand for the development of new environmentally friendly, fast, and cost-effective termite control technologies. Therefore, our proposal addressed the Statement of Need put forward by the SERDP in 2005, i.e., to develop innovative techniques that can help the DoDs resource managers in the area of invasive species control.

Termite symbionts as tools and targets for termite control: The workers of *C. formosanus* harbor a diversity of microorganisms (protozoa and bacteria) in their guts, which are necessary for the termites' survival. In particular the termite workers depend on three species of flagellate protozoa (*Pseudotrichonympha grassii* Koidzumi, *Holomastigotoides hartmanni* Koidzumi, *Spirotrichonympha leidyi* Koidzumi), which enable termites to degrade cellulose and digest wood efficiently (Bignell 2000). The use of paratransgenesis, genetically fortified indigenous species-specific microorganisms able to avoid immune detection and carry detrimental gene products, for insect control is an innovative but high-risk endeavor because various research trials and experimental approaches must be pursued to establish proof of concept and lay out the path for developing an environmentally- and user-friendly product. This project is likely to yield a high payoff, reducing costs of termite control and pesticide use. Paratransgenesis is likely to lead to a paradigm shift in termite control.

Previous studies: Husseneder and Grace (2005) isolated the bacterium *Enterobacter cloacae* from the gut of *C. formosanus* and genetically engineered this bacterium to express green fluorescent protein. The genetically modified bacterium was able to deliver and express foreign genes in the termite gut and was rapidly transferred among workers and soldiers in laboratory colonies (Husseneder et al. 2005a; and Husseneder and Grace 2005), providing proof of concept that paratransgenesis can be applied in termites. However, this shuttle system was only used as a tracer system and needed to be modified for termite control.

Lytic peptides have been shown to kill a wide variety of invertebrate protozoan parasites (Mutwiri et al. 2000; Hierath et al. 2002), and thus, are promising candidates to be used as toxicants against the symbiotic protozoa of termites. Lytic peptides, which are a ubiquitous part of the nonspecific immune system of eukaryotes, disrupt the membrane of microorganisms. Lytic peptides do not harm humans or other higher eukaryotes, because they do not affect the electrically neutral cholesterol-containing cell membranes of higher eukaryotes (Kamysz et al. 2003).

Previously, different types of synthetic lytic peptides (*agni, gagni, pagni* and *hecate*) were tested for their efficacy against *in vitro* cultures of the protozoa of *C. formosanus*. All peptides caused lysis of all protozoa species with varying potencies (Hierath et al. 2002).

However, no mortality was achieved when lytic peptides were fed directly to termites. Either, termites did not consume high enough doses of peptides or the peptides were inactivated by termite digestion (Jim Ottea, personal communication). Both pitfalls can be avoided by genetically engineering indigenous termite gut bacteria to express lytic peptides in the termite hindgut.

Advantage of paratransgenesis. The proposed novel approach of using microbes naturally associated with the target species to express protozoicidal lytic peptides in termites has major advantages over conventional pest control. Using termite specific bacteria to produce target specific toxicants, such as lytic peptides, is an environmentally friendly alternative to traditional chemical control. This system is a promising approach to eliminate termite colonies in a cost effective way, and would be a significant improvement over current baiting technology using insect growth regulators, because living bacteria in baiting systems can serve as a continuing source of inoculum, and are self-replicating, self-perpetuating and self-sustaining in the termite colony (Husseneder and Grace, 2005). Because they are not repellant and are able to bypass the immune barriers of termites, using indigenous bacteria from the termite gut as expression systems guarantees that they are readily introduced and spread throughout a termite colony by grooming and trophallaxis. Using recombinant bacteria for termite control has the advantage that molecular genetic techniques allow customization of the product in the future, e.g. optimizing the efficacy against the target species as well as environmental safety. For example, rate and amount of the gene product, and thus the speed of action, can be regulated by the choice of promoters. Field-life can be increased or suicide factors can be introduced to limit the life of the recombinant bacteria to minimize any environmental impact. Multiple genes can be inserted into the bacteria to avoid resistance and enhance effect on the target organism.

3. Materials and Methods

3.1 Task 1: Development of positive controls for testing lytic peptide performance

Subtask 1.1 Establish *Tetrahymena pyriformis* cultures

The initial positive control for testing the efficacy of lytic peptide solutions was the aerobic laboratory standard protozoa *Tetrahymena pyriformis,* cultures of which were purchased from Carolina Biological (#13-1182A) and maintained in proteose peptone media (Gorovsky et al. 1975). Before each experiment, lytic peptide activity was tested using *T. pyriformis*.

Subtask 1.2 Establish protozoa cultures from the gut

The three protozoan species (*Pseudotrichonympha grassii* Koidzumi, *Holomastigotoides hartmanni* Koidzumi and *Spirotrichonympha leidyi* Koidzumi) of the termite gut were maintained for 24 hrs *in vitro*, allowing time for testing of lytic peptides, following the optimization of a method outlined by Trager (1934). In an anaerobic glove-box, termite protozoa are taken from the termite hindgut and put into sterile, sparged (Hydrogen 2.5%, Carbon dioxide 5% and Nitrogen 92.5%) Trager U (pH 7.0).

Subtask 1.3: Determine the effects of defaunation of termites using Metronidazole

Using an aspirator, six groups of 100 termite workers were collected and placed in Petri dishes containing damp filter paper. In addition, soldiers were added to each group as higher termite survivorship has been recorded where 10 % of the populations are from the termite soldier caste (Maistrello et al. 2005). Three groups of termites were transferred to labeled Petri dishes containing filter paper treated with 400 µl Metronidazole (2 g/L) (Raina et al. 2004). The remaining three groups of termites were transferred to labeled Petri dishes containing paper dampened with autoclaved tap water. All six replicates were transferred to an incubator and mortality was recorded every 24 hrs. The termites were fed with their assigned treatment for seven days. After seven days five termite worker guts from each group were extirpated and the presence/absence of protozoa recorded under the microscope. Once defaunation was confirmed all six groups were provided with only filter paper dampened with sterile tap water. Mortality was recorded every day. Statistical difference in mortality in treatment groups and control groups was established using 95% confidence intervals.

3.2. Task 2: Evaluation of lytic peptides

Lytic peptides are charged proteins which will adsorb to charged surfaces, e.g., glass. When testing lytic peptides against protozoa this “sticking” reduces efficacy of the lytic peptide. Theoretically, exposure of protozoa to low concentrations of peptide may result in the observation that the peptide was not effective at killing protozoa, when in reality it was simply bound to the side of the tube and thus unavailable to bind to its intended target. Therefore, Sigmacote™ (Sigma, # SL-2) was used to treat all the glass and plastic-ware prior to use with lytic peptides. Sigmacote™ reacts with surface silanol groups on glass to produce a neutral, hydrophobic microscopically thin film. This neutral film prevents adsorption of basic proteins, such as lytic peptide, to the surface of the glass.

Subtask 2.1 Optimize the microinjection system

Efficacy of lytic peptide solutions were confirmed using *T. pyriformis* and termite protozoa cultures *in vitro* before each microinjection. A 5 ml overnight culture of *T. pyriformis* (subtask 1.1) was washed three times in sterile 10 mM Tris-HCl (pH 7.4) and suspended in 1 ml 10 mM Tris-HCl (pH 7.4). Subsequently, 100 µl volumes were placed in a Sigmacote™ treated 96 well Microplate. The ranges of concentrations (25-500 µM) of lytic peptide to be tested were added to five of the wells and the remaining wells were treated with 10 mM Tris-HCl (pH 7.4). The lytic peptides were suspended in 10 mM Tris-HCl because other buffers, such as Phosphate Buffered Saline, had been shown to adversely affect protozoa and reduce efficacy of lytic peptide when compared to their suspension in 10 mM Tris-HCl (pH 7.4). After confirming aerobic activity of the lytic peptide concentrations was *T. pyriformis*, lytic peptide efficacy was tested against anaerobic cultures of termite protozoa. Sparged lytic peptide was added anaerobically to the sparged cultures of termite protozoa (subtask 1.2) and mortality was observed.

The microinjection system was further optimized by determining the optimum amount of time needed to immobilize a termite through cooling on ice; identification of suitable methods of anchoring the termite in place for the microinjection and establishing the maximum volume to be injected without causing the death of the termite.

Subtask 2.2 Test delivery system of lytic peptides *in vivo*

Following completion of subtask 2.1 lytic peptides were delivered to the hindguts of termites. Termite workers were immobilized by chilling on ice for 1.5 min and mechanically aspirated head first into a modified pipette tip (termite holder), which had been cut to allow the terminus of the termite to be exposed. The termite holder was attached to a micromanipulator and the insect was advanced in a manner which inserted a Sigmacote™ treated glass needle into the anus of the termite. The injection was then made by use of a high-speed electronic foot pedal with a pulse length control, which insured that a constant volume was reproducibly injected. Approximately 0.5 µl 10 mM Tris-HCl (pH7.4) (control) or lytic peptide suspended in 10 mM Tris-HCl (pH7.4) was injected into the hindgut of the termite. A second control group consisting of non-injected termite workers was included. The termites were housed in a 3 cm Petri dish, damp filter paper was provided. At 24, 48 and 72 hrs guts from each treatment were extirpated, and observations regarding presence and motility of protozoa were made.

Subtask 2.3 Encapsulate lytic peptide

Biodegradable microspheres (Okada and Toguchi, 1995) consisting of poly(D,L-lactide-co-glycolide) (PLGA), which is a biocompatible, bioabsorbable, and biodegradable polymer, have been used to formulate many types of implantable and injectable drug delivery systems for humans and other animals. PLGA microspheres have been reported as carriers for site-specific delivery of various drugs like adapalene and tetracycline. By encapsulating the peptides in microspheres, such as those produced using the RESOMER® line of products from Boehringer Ingelheim, it may be possible to deliver a steady supply of lytic peptide over time to the termite hindgut. If microinjection of lytic peptide into the termite gut would not kill the hindgut protozoa, proteolytic digestion of the peptides would be the likely reason. In this case, encapsulation using lytic peptides would provide an alternative to microinjection. However, since injected lytic peptides were active in the hindgut encapsulation was not necessary (no go).

3.3 Task 3: Optimization of vector system (proof of concept)

Proof of concept for peptide expression and secretion was achieved using a commercially available yeast-based expression system in *Kluyveromyces lactis* (#E1000S) from New England BioLabs (NEB). The gene of interest is inserted into the yeast's genome where the protein is produced in a pro-form. The pro-protein is cleaved by the internal cell mechanisms and the active protein is secreted into the yeast's growth media, thus mimicking the proposed design of the gene-shuttle. Cloning the genes into *K. lactis* was performed using the manufacturer's protocols (Appendix C)

Subtask 3.1 Gene synthesis

Lytic peptides were selected for gene synthesis based on their efficacy determined in subtasks 2.1 and 2.2. Melittin (78bp) and Hecate (66bp) gene sequences were codon optimized for expression in *K. lactis* by Genscript Ltd. (http://www.genscript.com) and it was confirmed that DNA base changes did not result in amino acid substitutions. Fusion genes consisting of a lytic peptide fused at the genetic level to green fluorescent protein (subtask 4.1) were also codon optimized. The genes were then synthesized by Genscript (www.genscript.com) and cloned into a plasmid (pUC 57) and shipped in a lyophilized form. The plasmids were re-suspended in sterile water and used in accordance with NEB's *K. lactis* protein expression kit manual to produce lytic peptide expressing yeast strains. Integration was confirmed in accordance with the manufacturer's protocols (Appendix C).

Subtask 3.2 Develop controls for the yeast

Several control strains of *K. lactis* were engineered (subtask 3.3). The aim was to establish whether any specific observations during the feeding experiments were due to the presence of the yeast in the termite gut or were a direct result of the secretion of lytic peptide. The controls generated were *K. lactis* expressing Maltose Binding Protein (MBP), a non-toxic protein and *K. lactis* with the vector inserted, which would not secrete any protein.

<u>*Tetrahymena*/Yeast toxin activity assay</u>

K. lactis yeast strains engineered with lytic peptide genes and appropriate controls (*K. lactis* vector and Maltose Binding protein) were grown in 2 ml of YPGal in Sigmacote™ treated (Sigma-Aldrich #SL2) tubes for 2-3 days at 30°C, shaking at 225 rpm. In the meantime, a 15 ml glass tube with 5 ml Proteose Peptone media was used to grow a culture of *T. pyriformis* incubated at 30°C shaking at 100 rpm overnight. A 1 ml aliquot of the overnight culture was added to 50 ml of YPGal and incubated at 30°C shaking at 100 rpm overnight to increase the number of *T. pyriformis*. The yeast cultures (on the third day) were pelleted and the supernatant was retained for the assay. A Sigmacote™ treated 96 well cell culture plate was prepared with 50 µl of *T. pyriformis* in each well and an equal volume of yeast supernatant was gently mixed with the *T. pyriformis*. Extra wells of *T. pyriformis* without supernatant treatment were set up as more controls and to get an initial count. Cells were counted using a Hemocytometer and at 24, 48 and 72 hrs. Statistical analysis was undertaken using SAS Proc Mixed ANOVA model with Tukey's mean separation.

Subtask 3.3 Perform termite feeding experiments

Uptake of yeast through termite feeding

A termite feeding assay with fluorescently labeled (non-lethal fluorescent yeast vacuole stain, MDY-64, Sigma) was designed to determine whether yeast would enter and persist in the hindgut of termite workers. Twenty droplets of stained yeast were aliquoted in a circular arrangement in a clean Petri dish; a control dish with water droplets was also set up. Twenty termite workers were placed in each dish and after 1 hr termite guts were extirpated from each treatment and the presence of labeled yeast was confirmed using a Green Fluorescent Protein (GFP) microscope.

Termite feeding experiments

Kluyveromyces lactis strains that showed toxicity towards *T. pyriformis* (Subtask 3.2) were used in termite feeding experiments. Aliquots of 2 ml YPGal in Sigmacote treated 15 ml Falcon tubes were inoculated and grown for 3 days, 225 rpm at 30ºC. The strains grown were *K. lactis* engineered with the expression vector (control), *K. lactis* expressing the control protein MBP and *K. lactis* expressing a lytic peptide gene. The cultures were washed three times with sterile dH_2O and finally re-suspended in 500 µl sterile 10 mM galactose. The yeast cells were suspended in 10 mM galactose because the genes were under control of the LAC4 promoter (Colussi and Taron, 2005), activity if which could be enhanced by the presence of the sugar.

Termites were routinely collected from New Orleans and maintained on damp cardboard in plastic buckets at room temperature. Only termites that had been in the laboratory less than 4 weeks were used in the termite feeding experiments and a representative sample of the individuals were dissected and their guts observed to confirm health of the termite protozoa. Once the termite colony's guts had been confirmed as healthy, 28 groups of 50 termite workers and 5 termite soldiers were collected using aspirators. The groups were temporarily housed in Petri dishes on damp tissue paper covered with aluminum foil to allow the treatment dishes to be set up. The treatment dishes were subsequently assembled in clean, labeled Petri dishes. In a circular arrangement 20 distinct 2 µl droplets of the assigned yeast/control treatment were aliquoted into the appropriate dish. Four replicates of each treatment were set up: *K. lactis* expressing lytic peptide; *K. lactis* expressing MBP, *K. lactis* engineered with vector only; 10 mM Tris and eight dishes of sterile dH_2O. The groups of termites collected previously were randomly assigned to a treatment dish and gently transferred to the center of the droplets with a soft paintbrush. The dishes housing the termites were placed on damp tissue paper and covered with aluminum foil for 24 hrs. The next day all the termites were transferred to correspondingly labeled, clean Petri dishes and provided with filter paper dampened with 10 mM galactose. Four of the eight sterile dH_2O treatment dishes were provided with sterile dH_2O dampened filter paper, thus controlling for effects of 10 mM galactose on termites.

Termite mortality was recorded every three days. The viable number of termites was also recorded as cannibalism in laboratory colonies has been observed (unpublished data). Once per week termite guts were extirpated from two termites from each Petri dish and the activity and presence/absence of protozoa species were observed. For the duration of the experiment the termites in the Petri dishes were maintained on damp tissue paper and covered with aluminum foil. Differences in mortality considered significant if the 95%confidence intervals did not overlap.

3.4 Task 4: Lytic peptide mode of action

Subtask 4.1 Develop GFP-Lytic peptide fusion genes

Lytic peptide genes were fused at the genetic level to a GFP gene (subtask 3.1), optimized for expression in yeast and synthesized by Genscript Ltd. The fusion-genes were used to monitor expression levels using anti-GFP antibodies and to observe action of the lytic peptide inside the termite. The lytic peptide:GFP genes were cloned into *Kluyveromyces lactis* and expression was confirmed using a *T. pyriformis* bioassay. Subsequently, the strains causing protozoa death were used in termite feeding experiments.

Lytic peptide:GFP fusion protein activity in the termite hindgut was to be observed using a GFP microscope with excitation of the GFP moiety at 395 nm and emission in the lower green portion of the visible spectrum (509 nm). Two groups of 20 termite workers were transferred into clean Petri dishes. Each group was fed with either 20 discrete 2 µl droplets of water or with *K. lactis* expressing lytic peptide:GFP, following a 24 hr feeding period termite guts were extirpated and fluorescence was to be observed under a GFP microscope. However, expression levels in the termite gut were too low to be detected by a visual signal and the mode of action of lytic peptides was not revealed.

Subtask 4.2 Identify receptors

A receptor, to which a ligand could be developed and fused to the lytic peptide, would ensure specific targeting of the peptide to the protozoa, thus introducing a termite specific mode of action. The presence of insulin receptors was investigated by treating termite protozoa with fluorescently labeled Insulin (Invitrogen) and observing internalization of the insulin using a GFP microscope. The presence of the insulin receptors was originally supposed to be investigated using *in situ* hybridization techniques in collaboration with the LSU microscopy suite; however a commercially available kit from SIGMA (#PI0100-1KT) was used instead.

The kit was a solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA) design developed by SIGMA. A monoclonal capture antibody specific for the insulin receptor (IR) (regardless of phosphorylation state) was coated onto multi-well strips. Standard dilutions and samples were incubated for 2 hrs at room temperature. IR antigen was added and bound to the capture antibody. After a wash stage, a detection antibody specific for the non-phosphorylated or phosphorylated protein was added and the assay was incubated for 1 hr at room temperature, which results in binding to the immobilized IR protein. An anti-rabbit IgG-Horseradish Peroxidase (HRP) was added and subsequently bound to any immobilized protein present, completing the four-member sandwich. The reaction was visualized by adding tetramethylbenzidine (TMB) substrate, followed by the stop solution supplied with the kit. The intensity of the yellow color was measured in a multi-well plate reader at 450 nm and was directly proportional to the concentration of IR in the original sample. The unknown concentrations were calculated from the standard curve run with each assay.

3.5 Task 5: Construction of a termite specific gene product

Subtask 5.1 Detection of Enterokinase within the termite gut

We proposed to assemble a termite specific fusion gene that consisted of a secretion protein linked to a lytic peptide via a termite enzyme specific cleavage site. The final aim of using this fusion gene was to ensure environmental safety of the paratransgenesis system. A likely enzyme candidate with a unique protein cut site was Enterokinase. The termite gut was screened for the presence of Enterokinase using commercially available kits from NEB and SIGMA using SDS-PAGE and Western blotting.

Subtask 5.2 Construction of a test protein

The termite gut contents may degrade the test proteins supplied in the commercial kits, e.g., the general protease trypsin is common within insect guts. If this is the case, a synthetic, stable peptide containing the unique Enterokinase cut site will be developed. This subtask was not accomplished.

Subtask 5.3 Identification of general gut enzyme profile

Extra-cellular enzymes (proteases) were isolated from cell free culture supernatant and the activity of the proteases using SDS-PAGE electrophoresis with different embedded protein substrates. Proteases were partially sequenced for identification and construction of their specific cleavage sites. The following fractions of the termite gut were collected anaerobically from termite worker guts for proteolytic investigations: whole termite guts; washed/empty guts; gut contents and finally washed protozoa. During collection the samples were kept on ice at all times and stored at -20 ºC to prevent protein degradation.

Sample preparation procedure for each fraction:

Whole termite guts: Guts were extirpated from 20 termite workers and pooled in a clean 1.5 ml microcentrifuge tube with 200 µl of Tissue-PE LB buffer (GBiosciences). While on ice, the guts were homogenized with a sterile micropestle and centrifuged at 3000 rpm for five minutes. The supernatant was discarded and the homogenized guts were re-suspended in 200 µl of fresh Tissue-PE LB buffer.

Washed/empty gut: A clean Petri dish was placed on ice to cool and 20 ml 10 mM Tris (pH 7.4) was added. Guts were extirpated from 20 termite workers, pierced and washed in the 10 mM Tris. Each of the washed guts were put into a 1.5 ml microcentrifuge tube with 200 µl of Tissue-PE LB buffer and homogenized gently with a sterile pestle and centrifuged at 3000 rpm for five minutes. The supernatant was discarded and the empty guts were re-suspended in 200 µl of fresh Tissue-PE LB buffer.

Gut contents: A 10 µl droplet of Tissue-PELB buffer was placed in a cooled Petri dish and an extirpated termite gut was placed into the droplet and pierced to release the contents of the gut. The gut wall was discarded and the remaining gut contents/droplet was placed into a microcentrifuge tube. This was done 20 times using 20 termite worker guts.

Protozoa: A 10 µl droplet of Tissue-PELB buffer was placed in a Petri dish. A termite gut was extirpated, placed into the droplet and pierced to release the contents of the gut. The gut wall was discarded and the remaining gut contents/droplet was placed into a microcentrifuge tube. This was repeated with 20 guts in total. Subsequently, 1 ml of Tissue-PELB was added to the gut contents and centrifuged at 3000 rpm for five minutes. The supernatant was discarded

and the pellet was re-suspended in 200 µl of Tissue-PELB buffer.

After dissecting, the samples were again homogenized (on ice) using a sterile pestle being careful not to raise the temperature. The samples were then centrifuged at 13,000 rpm for 30 minutes at 4 °C. The supernatant was saved and stored at -20 °C until a Zymogram gel was performed.

Zymogram gels protocol:

Samples were allowed to thaw on ice to minimize protein degradation and were mixed with Zymogram Sample Buffer (one part sample to two parts sample buffer (Bio-Rad #161-0764)). The protein standards used were pre-stained SDS-PAGE Standards, broad range (Bio-Rad #161-0318) and Precision Plus Protein All Blue Standards (Bio-Rad # 161-0373). The standards were denatured at 85C for 10 minutes before being loaded into the gel (Criterion, Zymogram gel, casein 12.5 %). Electrophoresis of the samples was at 100 V for 2-2 ½ hours in ice cold running buffer as well as the gel tank being submerged in ice water. The gels were then later incubated in 100 ml Zymogram Renaturation Buffer (Bio-Rad #161-0765) on ice for 30 minutes with gentle agitation changing the buffer about every 10 minutes. After renaturation, the gels were placed in 1x Zymogram Development buffer (pH 7.8 (Bio-Rad #161-0766)) for 30 minutes to equilibrate to room temperature, and then placed into fresh buffer to incubate at room temperature for 3 hours. In other experiments, the development buffer pH was altered (pH 5.0, 7.0, and 9.0) as well as the incubation temperatures (room temperature, 30°C, and 37°C). When the development was finished, the gels were stained with Coomassie Blue R-250 (Bio-Rad #161-0400) overnight at room temperature with gentle agitation. The following day the gels were de-stained for 30-45 minutes checking the gels every 15 minutes. The gels were dried using GelAir Drying system (Bio-Rad #165-1771).

The experiments were repeated using 10 µl volumes of gut samples as well as looking at the effects of different protease inhibitors against the samples. The inhibitors used were EDTA (ethylenediaminetetraacetic acid, 8 mM), a metalloproteases inhibitor, and PMSF (phenylmethylsulfonyl fluoride, 10 mM), a serine protease inhibitor, both of which were purchased from Sigma Aldrich (#EDS-100 g and #P7626-1 G respectively). Controls for the inhibitors were the enzymes 1 unit/µl thermolysin (Sigma #P1512), 10 units/ml carboxypeptidase-A (Sigma #C9268), 500 BAEE units/ml trypsin (Sigma #T4799), 5 U/ml chymotrypsin (Sigma #C4129) and 1 U/mg papain (Sigma #P3250).

SDS- PAGE electrophoresis protocol:

Tris-HCl 12.5 % gradient gels were purchased from BioRad. Protein samples were thawed on ice then mixed with an equal volume of Laemmeli sample buffer. The protein samples and markers were then incubated at 85 ºC for 10 minutes and put back on ice. During this time the Tris-HCl gel was placed in the electrophoresis rig and covered in 1x Tris-HCl running buffer. The samples and markers were loaded and run for 1-2 hrs at 120V. The gels were stained overnight in coomassie blue G250 and then de-stained for 3 hrs changing the de-stain buffer every hour. Protein bands were cut out of the gel and sent to the University of Florida for LC-MS sequencing.

3.6 Task 6: Transformation of indigenous gut bacteria

Subtask 6.1 Growth curve development of *Pilibacter termitis*

A 5 ml Brain Heart Infusion (BHI) broth was inoculated with an isolated *P. termitis* colony and held under anaerobic conditions for 24hrs at 30ºC. A 100 µl aliquot of culture was used to inoculate a fresh 5ml broth and grown for 24 hrs at 30ºC. At 0, 1, 4, 8, 12, 18 and 24 hr, a 100 µl aliquot was removed and diluted. Dilutions were spread on BHI plates and following 24 hrs incubation the colony forming units (cfu) determined for each time point. After three repetitions of the experiment the mid-exponential phase of growth at 30 ºC was determined to be at 18 hrs. Subsequently, all transformations were conducted on bacterial cultures of 18 hrs.

Subtask 6.2 Transformation of *Pilibacter termitis*

Copious numbers of transformation protocols were outlined in the literature; many electroporation conditions available for investigation. Methods employed by Siegel et al. 1981; Wickner and Chassy, 1984; Nickoloff, 1995; Trevors et al. 1992; Jeltsch and Pingoud, 1996; Kobayashi, 2001; Chassy and Flickinger, 1987; Stratz et al. 1993; Chassy and Giuffruda, 1980; Chassy et al. 1988 and Holo and Nes, 1989 were used to guide choices in the variety of plasmids and transformation parameters. This subtask was not successfully completed and thus, task 7 could not be achieved.

3.7 Task 7: Optimization of gene constructs for bacteria

The gene constructs were to be optimized for bacteria that have been isolated from the termite gut (Task 7). The proposed expression vector for the gram-positive *Pilibacter termitis* was based on integration vectors available for *Bacillus subtilis*, the vector for the gram-negative *Dysgonomonas* spp. was based upon *E. coli* expression systems. As soon as indigenous gut bacteria were successfully transformed and the expression of lytic peptides was confirmed using the positive controls (subtask 1.2), bioassays were to be conducted to determine the effects of genetically engineered bacteria on the survival of the protozoan symbionts of termites and ultimately the survival of termites. This task was not completed.

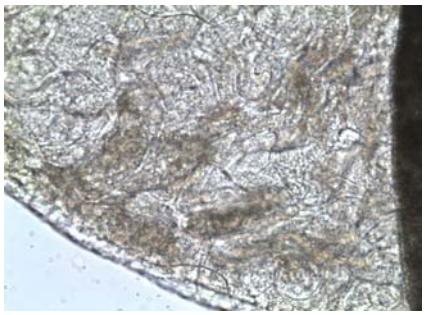
4. Results and Accomplishments

	Task		Subtask	Completion Status
1.	**Development of positive controls for testing lytic peptide performance**	1.1	Establish *Tetrahymena pyriformis* cultures	**Completed**
		1.2	Establish protozoa cultures from termite gut	**Completed**
		1.3	Determine effects of defaunation of termites using Metronidazole	**Completed**
2.	**Evaluation of lytic peptides**			
		2.1	Optimize the microinjection system	**Completed**
		2.2	Test delivery system of lytic peptides *in vivo*	**Completed**
		2.3	Encapsulate lytic peptides	**Not necessary**
3.	**Optimization of vector system (proof of concept)**			
		3.1	Gene synthesis	**Completed**
		3.2	Develop controls for the yeast	**Completed**
		3.3	Perform feeding experiments	**Completed**
4.	**Lytic peptide mode of action**			
		4.1	Develop GFP-Lytic peptide fusion genes	**Completed**
		4.2	Identify receptors	**No insulin receptors found**
5.	**Construction of termite specific gene product**			
		5.1	Detection of Enterokinase in termite gut	**Completed, no EK found**
		5.2	Possible construction of test protein	**No go**
		5.3	Identification of general gut enzyme profile	**Completed, but no unique enzymes found**
6.	**Transformation of indigenous gut bacteria**			
		6.1	Establish growth curves	**Completed**
		6.2	Establish electroporation conditions	**Unsuccessful**
7.	**Optimization of gene constructs for bacteria**			**Uncompleted dependant on 6.2**
		7.1	*Pilibacter termitis* gene expression	**Uncompleted**
		7.2	*Dysgonomonas* spp. gene expression	**Uncompleted**
		7.3	Transformation with lytic peptide gene constructs	**Uncompleted**
		7.4	Conduct bioassays	**Uncompleted**
8.	**SERDP Reporting**			
		8.1	Submit Completed Fact Sheet	**Completed**
		8.2	Submit Final Report	**Completed**

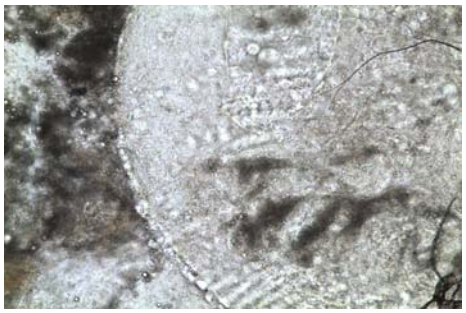
Figure 1. Completion status of tasks and subtasks

4.1 Task 1: Development of positive controls for testing lytic peptide performance

Positive controls for the testing of lytic peptides were established. Efficacy of lytic peptides were routinely tested prior to experiments to confirm activity aerobically using *T. pyriformis* (subtask 1.1) and anaerobically using cultures of termite protozoa (subtask 1.2). When treated with 50 µM concentrations of the lytic peptides, 100% mortality was observed in 5-10 minutes, thus confirming toxicity of the three lytic peptides (Hecate, Melittin and Cecropin) towards termite protozoa in an anaerobic environment, mimicking termite hind-gut conditions. The effect of gut defaunation on termite survival (subtask 1.3) was determined using the protozoicidal drug Metronidazole (2 g/L), which kills protozoa (Raina, et al. 2004). Defaunation of termite workers was observed at 7 days (Figure 2.) and the defaunated termites died within six weeks, while the untreated termites were not defaunated and did not die (Figure 2.). Therefore the first task of developing positive controls for testing lytic peptide performance was successfully completed. The completion of task 1 provided positive controls for the evaluation of lytic peptide action in task 2, the development and use of the microinjection system.



Untreated termite gut (protozoa present)



Treated termite gut (defaunated)

Figure 2. Defaunation of the termite hindgut.

After seven days of termite workers being fed Metronidazole the three species of protozoa in their guts were dead (defaunation)

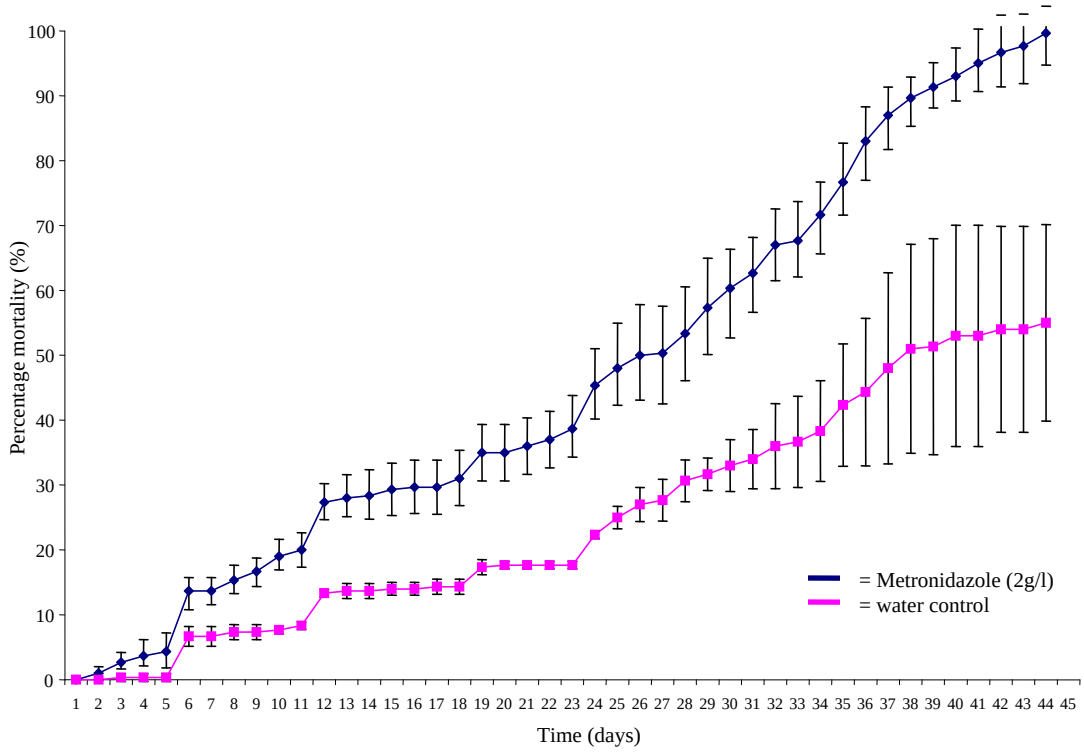


Figure 3. Mortality of termites workers after defaunation with Metronidazole.

Defaunation occurred at 7 days of feeding with Metronidazole. Defaunation led to death of workers within six weeks. Each treatment consisted of three replicates; confidence intervals of 95% did not overlap and thus showed significant higher mortality of defaunated termites versus controls.

4.2 Task 2: Evaluation of lytic peptides

The microinjection system (subtask 2.1) was optimized and became a reliable method of administering lytic peptides to the termite hindgut, which might otherwise be degraded by non-specific protease activity in the termite foregut and midgut. Using microinjection, it was shown that 50 µM concentrations of lytic peptides defaunate termites within 72 hrs of treatment (subtask 2.2). It should be noted that protozoicidal effects are observed in the microinjection system at a slower rate than in the *in vitro* assays, 72 hrs vs. 5–10 minutes, because the volume of peptide injected into the hindgut was limited to ~0.5 µl. From these experiments the most effective peptides against the protozoa *in vivo* were determined to be Hecate and Melittin. Due to the success of the microinjection system, encapsulation of lytic peptides for feeding experiments was not necessary and subtask 2.3 was considered "no go".

4.3 Task 3: Optimization of vector system (proof of concept)

A prototype expression system for proof of concept that paratransgenesis in termites could work was required. The lytic peptides Melittin and Hecate were selected as the toxins for the proof of concept. The genes were codon optimized for yeast expression and chemically

synthesized (Genscript) and subtask 3.1 was completed. The genes were cloned into yeast, *Kluyveromyces lactis*, using the pKLACI vector (NEB). Transformation of the *K. lactis* with the vector plus gene of interest was implied by the growth of *K. lactis* on defined manufacturer's growth media containing acetamide as the only Nitrogen source and was confirmed using the supplier's polymerase chain reaction (PCR) primers (NEB). A 1.9 kb bp PCR product was an indicator that insertion was successful. Controls were concurrently developed while cloning the peptide gene into *K. lactis* to complete subtask 3.2. The controls included a strain of *K. lactis* secreting Maltose Binding Protein (MBP) and *K. lactis* containing the pKLACI vector but no protein encoding gene; transformation was also confirmed. Secretion of the MBP control protein was confirmed through a Western Blot system developed by the manufacturer (Appendix C). Secretion of active lytic peptide by the transformed *K. lactis* into the growth media was verified using *in vitro* cultures of the protozoa *T. pyriformis*. Growth media of two strains of *K. lactis*, one engineered to secrete Hecate and the other Hecate-GFP, produced high enough levels of the peptide to result in a significant higher mortality of *T. pyriformis* at 72 hrs than the controls (SAS Proc Mixed ANOVA, Tukey's mean separation, Figure 4.).

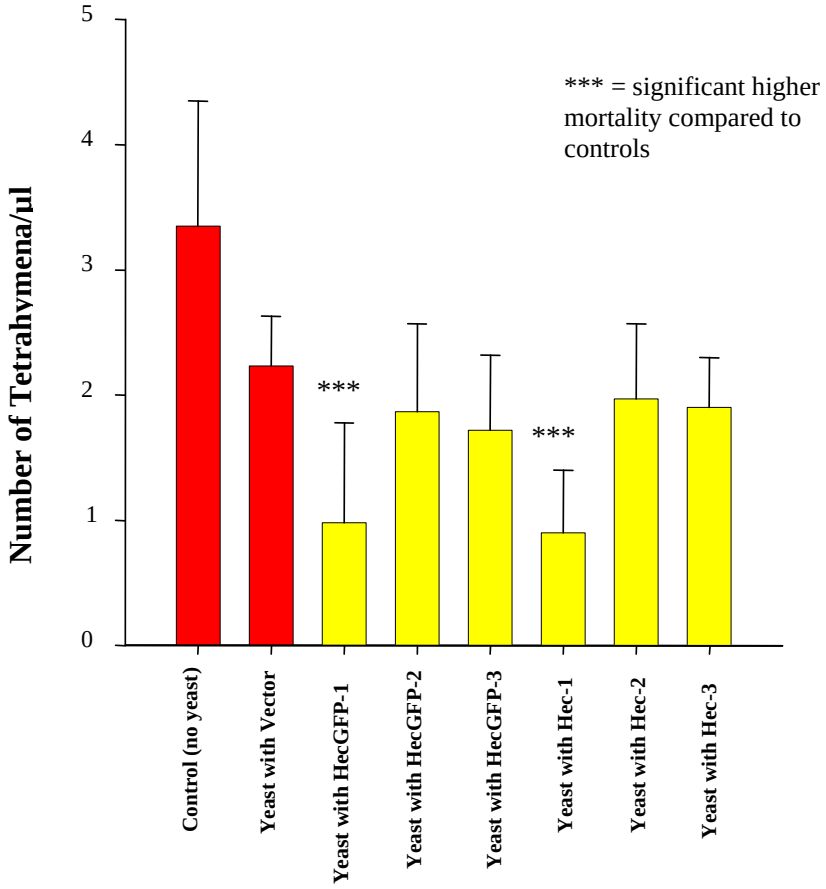


Figure 4. Confirmation of lytic peptide expression using a *Tetrahymena pyriformis* bioassay. The number of *T. pyriformis* present at 72 hrs after treatment with supernatant of genetically engineered yeast strains and in controls are shown with their standard error bars (4 replications).

As a consequence of the production and confirmation of "killer-yeast-strains" defaunation experiments were begun. Groups of termites were fed with one of the following: (1) Hecate-expressing *K. lactis*; (2) Hecate-GFP expressing *K. lactis*; (3) MBP expressing *K. lactis*; (4) non-expressing *K. lactis* (vector only); (5) 10 mM galactose; or (6) sterile water (subtask 3.3). The numbers of termites alive and dead were recorded every three days and observations on the presence and activity of termite protozoa in the hindgut were made every seven days. Termite feeding experiments with the peptide expressing yeast resulted in defaunation of termites at four weeks. However, when feeding experiments were repeated four weeks later to measure termite mortality, the mortality in termites treated with lytic peptide expressing genes was not significantly higher than in the controls (Figure 5.).

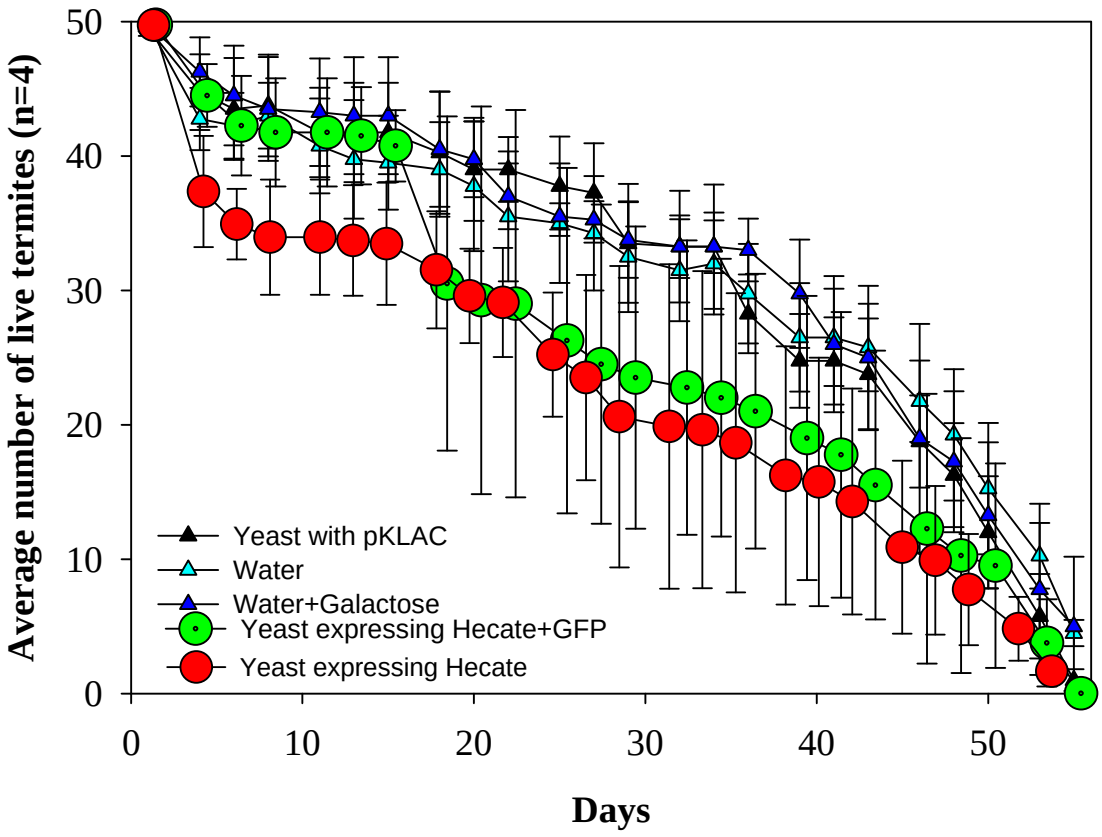


Figure 5. Termite mortality in feeding experiments. Overlapping 95% confidence intervals show no significant difference between mortality of termites in the controls and termites fed yeast engineered to express lytic peptides approximately two months after the yeast strains were first engineered.

Upon replication of the termite feeding experiment using a different termite colony no defaunation was observed, contrary to previous results. Similarly, re-testing of supernatants of the previously confirmed "killer-yeast-strains" on *T. pyriformis* cultures, did not lead to cell death anymore. Therefore, it was concluded that the killer strains were not expressing active lytic peptide ~8 weeks after first confirmation of lytic peptide toxicity using a *T. pyriformis* bioassay.

During consultation with the *K. lactis* NEB project managers, recombination events in the *K. lactis* genome experienced by other research groups working with this system were discussed. The recombination events had resulted in loss of their *K. lactis* gene expression. Following several failed repetitions of the PCR used to confirm integration of the vector and gene into the *K. lactis* genome, we concluded that genomic recombination had most likely resulted in the loss of gene expression in our system. Although tolerance of *K. lactis* against lytic peptides in the media had been established prior to selecting this yeast as a microbial host, expressing and secreting lytic peptides obviously puts selective pressure on the yeast. While this loss of expression over time has the advantage of limiting environmental impact, further investigation into storage of yeast stocks (e.g., lyophilization) is necessary, before a yeast-based bait system for termite control can be developed.

Nevertheless, proof of concept was achieved that lytic peptide expressing yeast defaunates termites and subtask 3.3 was thus completed. Efforts to reproduce the killer-yeast-strains are ongoing. Western Blots were optimized to detect ~2 ng pure protein. With this sensitivity we will be able to quantify the amount of Hecate and Melittin being secreted from the yeast once successfully engineered. With the optimization of Western blot techniques, it should possible to confirm persistence of expression of lytic peptide *in situ*.

4.4 Task 4: Lytic peptide mode of action

Investigation of the lytic peptide mode of action involved the development of a lytic peptide:GFP fusion gene and its subsequent observation within the termite hindgut (subtask 4.1). Subtask 4.1 was initiated by producing fusion genes through the synthesis of lytic peptide genes joined to GFP genes, both of which were codon optimized for expression in yeast by Genscript. The Melittin:GFP and Hecate:GFP were both cloned into *K. lactis*. Transformation of lytic peptide:GFP was confirmed using PCR. Western Blots did not detect sufficient expression of Melittin:GFP. However, expression of Hecate:GFP in *K. lactis* strains were verified using a *T. pyriformis* bioassay (Figure 4).

The *K. lactis* strain expressing Hecate:GFP fusion proteins was subsequently fed to termite workers and the guts extirpated and observed under the GFP microscope. However, because Hecate:GFP is secreted from the yeast, it does not accumulate in high enough quantities to fluoresce at an observable level. Although subtask 4.1 was completed the mode of lytic peptide action was not revealed as the fusion proteins could not be detected in the termite gut by their fluorescence alone. In the future, labeled GFP antibodies will be used to detect fusion proteins in the termite gut.

Subtask 4.2 was to identify receptors on the surface of termite protozoa towards which a specific ligand could be designed. Ligand-linked lytic peptides would ensure specific targeting of the peptide to the protozoa. The presence of insulin receptors on the surface of termite protozoa was hypothesized after protozoa were observed internalizing fluorescent insulin (fluorescein isothiocyanate conjugate of human insulin, Invitrogen) under the GFP microscope. The presence of the insulin receptors was initially to be confirmed using *in situ* hybridization, once the equipment was been made available through the LSU microscopy suite, however a commercial kit, the phospho-Insulin Receptor β Subunit ($pTyr^{1158}$) ELISA (#PI0100-1KT), became available from Sigma. Insulin receptors were successfully identified on the positive control, *T. pyriformis*, however, no receptors were detected on the protozoa from the termite gut and, therefore, subtask

4.2 was not successfully completed. In the future, alternate receptors will be investigated. A recently constructed Formosan termite cDNA library contained expressed sequence tags suggesting the presence of chemoreceptors on the surface of the protozoa.

4.5 Task 5: Construction of a termite specific gene product

To construct a termite specific gene product that could be activated in the termite hindgut by a unique hindgut enzyme, initial investigations focused on the identification of the Enterokinase (EK) activity (Subtask 5.1). Enterokinase was chosen because of its specific cut site, EK only cuts at AspAspAspAspAspLys. Presence of EK was investigated using the commercially available EK cleavage kit (Novagen). The cleavage control protein (48 KDa) supplied in the kit was cleaved by EK into two fragments (32 KDa and 16 KDa). The smaller 16 KDa had an S-Tag attached to it allowing its detection via western blotting. Digestion of the control protein by EK could be monitored. While treatment of the cleavage control protein with EK led to a western blot signal at 16 KDa, treatment of the cleavage control protein with hindgut extracts did not. It was concluded that either the protein was completely degraded by general endo- and/or exo-peptidases in the hindgut or EK was not present. Subtask 5.1 was completed, but Enterokinase was not detected. As an alternative, the investigation of the general enzyme profile of the termite gut could result in the identification of unique termite gut enzymes (Subtask 5.3). Initially it was intended that if the termite gut contents could degrade the test proteins supplied in the commercial kits (the general protease trypsin is common within insect guts) a synthetic, stable peptide containing the unique Enterokinase cut site would be developed. However, by identifying the enzyme profile of the termite gut, termite specific enzymes could be isolated, so subtask 5.2 was not necessary and determined to be “no-go”. Using protease detection gels, known as Zymogram gels, which contain Gelatin as a protease substrate, termite gut extracts were analyzed for protease activity. Four types of sample were investigated: whole termite guts; washed/empty guts; gut contents; and washed protozoa. The presence of protease activity in the different fractions was confirmed and a general profile was obtained. To date, serine and metallo- proteases have been identified using selective inhibitors (Figure 6.).

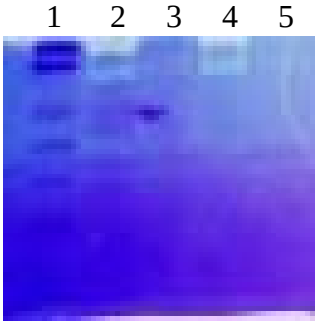


Figure 6. Zymogram of a serine protease and a metallo-protease from the termite gut

Lane 1 = Marker; Lane 2 = whole termite gut; Lane 3 = whole termite gut plus PMSF inhibitor (serine proteases); Lane 4 = washed termite gut and Lane 5 = washed termite gut plus EDTA inhibitor (metallo-proteases)

Twenty three proteins tentatively identified as proteases have been cut out of SDS-PAGE gels (Figure 7.) and sent to University of Florida for Liquid Chromatography- Mass Spectrometry (LC-MS) sequence determination. Only general trypsins have been found so far among those first protein sequences. Additional protein bands are currently processed to search for enzymes with a unique cleavage site. Also, several proteases were found in an expressed sequence tag cDNA library of Formosan subterranean termites and full gene sequencing in progress. At the time of submission of this report subtask 5.3 was not yet completed. When the identification of specific termite enzymes is complete the fusion gene will be constructed.

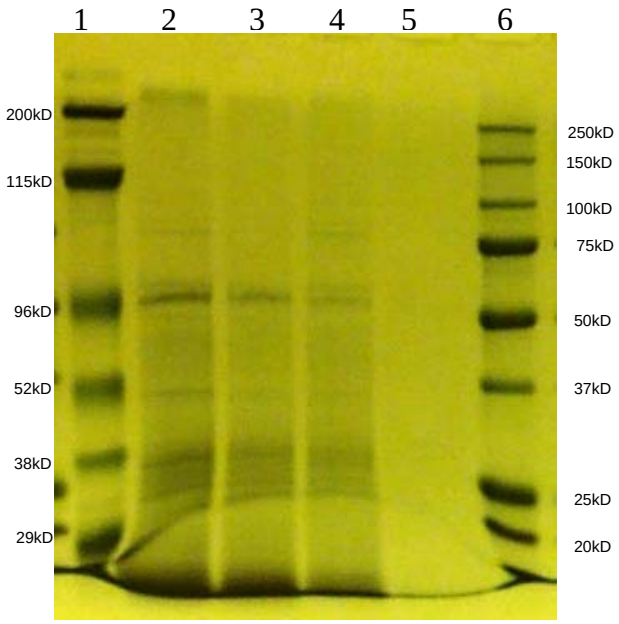


Figure 7. Protein profile of different components of the termite gut.

12.5% Tris-HCl protein SDS-PAGE gel: Lane 1= Protein marker; Lane 2 = whole termite gut; Lane 3 =washed termite gut; Lane 4 = termite gut contents; Lane 5 = washed termite protozoa; Lane 6 = protein marker.

Bands of 23 proteins putative proteolytic activity (see Figure 6) were cut out and sequenced.

4.6 Task 6: Transformation of indigenous gut bacteria

Additional environmental safety of a paratransgenesis system was to be provided by using novel bacteria that are exclusively found in the termite gut as expression systems. Candidate microorganisms were *Pilibacter termitis* (Higashiguchi et al. 2006) and two *Dysgonomonas* spp. which had been isolated from the termite hindgut (Husseneder et al. 2005b). Protocols for the genetic manipulation of these novel bacteria had not been established so investigations into the conditions needed for transformation were undertaken. *P. termitis* is a gram positive bacterium and lytic peptides kill gram positive bacteria more slowly than gram

negative bacteria, such as *Dysgonomonas* spp. Therefore, *P. termitis* was selected as the primary bacterial host.

Electroporation was selected as the mode of genetic manipulation (Trevors et al. 1992). Available literature recommended that transformation be performed on microorganisms in the mid-late exponential phase of growth (Trevors et al. 1992). The growth curve for the bacterium *P. termitis* was created (Figure 8.) and the mid-late exponential phase identified at ~18 hrs post inoculation. Subtask 6.1 was successfully completed.

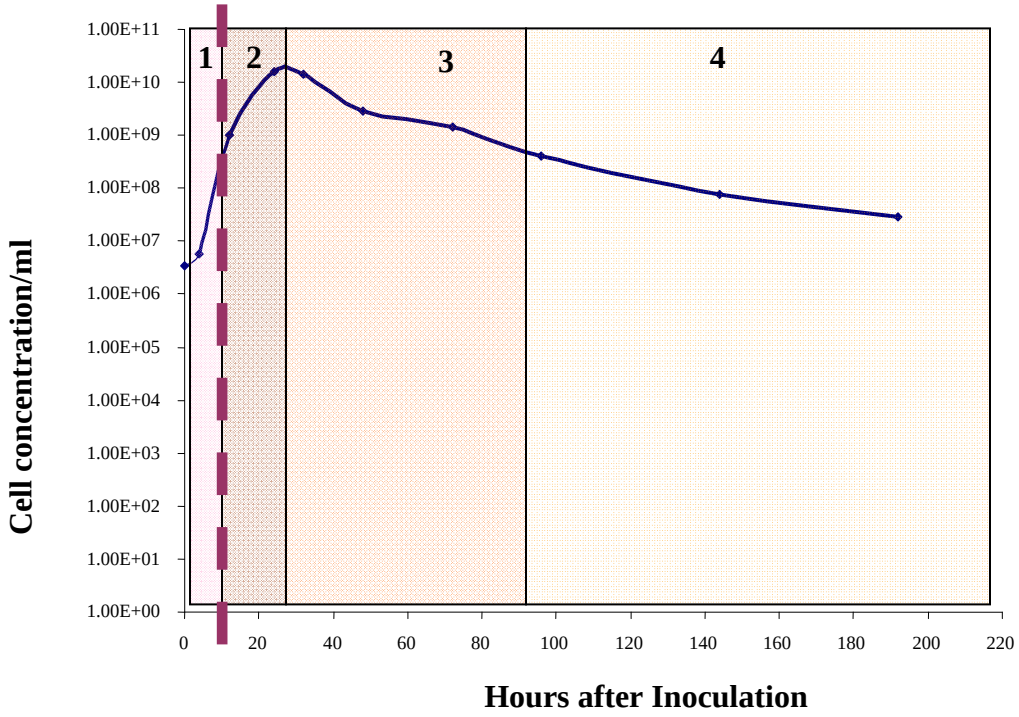


Figure 8. The growth curve at 30º C of the gram positive bacterium *Pilibacter termitis*
1= early exponential growth phase; 2 = mid-late exponential growth phase; 3 = stationary phase; 4 = death phase
- - = 18 hrs post inoculation is the preferred time for transformation of *Pilibacter termitis*

Protocols for the transformation of *P. termitis* did not exist in the literature as this bacterium is a novel species from the termite gut. Methods used on genetically similar microorganisms were used as a starting point for protocol development. Methods outlined by Chassy and Flickinger (1987) and Stratz et al. (1993) made use of cell wall synthesis inhibitors such as L- threonine (Chassy and Giuffruda, 1980; Chassy et al. 1988) and glycine (Holo and Nes, 1989), but when used with *P. termitis*, the additives did not yield transformants. Different electroporation buffers were tested including 1 M Sucrose and 0.5 M Sucrose/5 % glycerol at different voltages in combination with anaerobic recovery periods ranging from 1 hr-24 hrs;

these attempts also yielded no transformants. Various plasmids were tried in conjunction with the different conditions. For example the plasmid pDL278 was obtained from the University of Minnesota. This plasmid had previously been used to determine electroporation conditions of a *Streptococcus* sp., which was identified as being phylogenetically related to *P. termitis*, however no transformants were obtained.

Chemical methods of genetic manipulation of bacteria were reviewed and protoplast formation had been routinely used to engineer other microorganisms. The use of lysozyme and/or mutanolysin treatment of the cells to weaken the tough gram-positive cell wall in transformation technique has been well documented (Siegel et al. 1981; Wickner and Chassy, 1984; Nickoloff, 1995). Despite these efforts, the transformation of the novel termite-specific *P. termitis* has failed so far; subtask 6.2 was not completed. There were several factors contributing to the incompletion of the task, including: the unknown nature of the bacterium; growth medium used to culture recipient cells may influence efficiency of electroporation; the size of plasmid DNA that can be introduced may impose limits; effects of bacterial morphology; cuvette geometry and size may be specific to the microorganism; and problems associated with restriction endonuclease systems may exist (Trevors et al. 1992; Jeltsch and Pingoud, 1996; Kobayashi, 2001), i.e., without knowledge of the restriction endonuclease system in the bacterium, the introduced DNA may simply be digested. Further research into the genetic system of the novel indigenous termite gut bacteria, such as establishing the type of endonuclease system present, will be necessary to use bacterial gut symbionts as a termite specific gene shuttle. The proposed optimization of gene constructs for expression from indigenous termite bacteria (Task 7) was not completed. Completion of this task was dependant on the uncompleted subtask 6.2, the determination of electroporation conditions of indigenous gut bacteria.

While the conditions for the transformation of *P. termitis* remain elusive, it may be possible to transform another of the over 20 termite gut bacteria, which have been cultured so far (Husseneder et. al. 2005b). Also, yeast still remains a candidate host for the paratransgenesis system. The use of yeast as the host could result in the manufacture of a product that could be freeze-dried resulting in a long shelf life and be easily introduced to termite colonies in currently existing bait systems.

5. Conclusions

The Formosan subterranean termite damages military structures and training facilities in temperate, tropical, and subtropical regions. The Department of Defense has invested billions of dollars in wooden structures and products and termite management costs are second only to those for cockroach control. Invasive termites are easily transported around the world with movement of wooden material. Therefore, it is important to develop new environmental friendly long-lasting termite control technology, preferably without the use of chemical pesticides, to reduce termite damage to military installations, reduce the risk of spreading invasive termites with military movements, and lower the Department of Defense's costs for termite control and damage repair.

Many of the tasks detailed in this study, required for the production of paratransgenetic system to control termites, were completed as a result of SERDP funding. With completion of task 1 protozoa cultures were developed for confirming the protozoacidal activity of lytic peptides; also, the use of a protozoacidal drug proved that defaunation of the gut leads to the death of termites. With completion of task 2 a microinjection system was developed to deliver lytic peptides into the hindgut and it was shown that lytic peptides defaunate termite guts. With completion of task 3 a yeast-based prototype for a paratransgenic system was developed. Proof of concept was achieved that genetically engineered microorganisms, which express lytic peptides, are ingested by termites through feeding and subsequently defaunate termite guts. The yeast-based paratransgenic system lost lytic peptide expression after two months of repeated culture and needs thus to be freeze-dried for long-term storage. The natural loss of lytic peptide expression, however, is a useful "safety feature" to limit environmental impact.

While a prototype of a paratransgenic system for termite control was successfully developed some of the tasks that were meant to add additional environmental safety features have not been completed during the funding period. Insulin receptors (task 4) were not detected on the surface of the protozoa in the termite gut. Thus, other surface receptors, such as chemoreceptors, have to be investigated before ligand-linked lytic peptides can be developed that specifically target membrane receptors of the termite gut protozoa. Also, the search continues to identify the unique cut site of a termite-specific enzyme that could be used to activate lytic peptide pro-toxins (task 5). While serine and metallo-proteases have been detected in various components of gut, gut fluid and/or protozoa, protein sequencing has not yet revealed enzymes with unique cleavage sites. More putative proteases have to be sequenced and identified.

While a yeast-based paratransgenic system is probably the easiest to apply in form of baits, since yeast can be easily cultured and freeze dried for storage, we originally proposed to engineer termite specific gut bacteria, which are unlikely to survive in the environment for a prolonged time. Although the recently described novel lactic acid bacterium *Pilibacter termitis* seemed like the ideal microbial host candidate, because of its ubiquitous distribution in Formosan subterranean termite colonies from around the world and its natural tolerance against lytic peptides, genetic transformation failed despite all efforts. The other ubiquitous bacteria (*Dysgonomonas* spp) that were identified in the original proposal as potential candidates to become a microbial host were less resistant against lytic peptides than the gram-positive *Pilibacter* and were therefore not transformed. Experiences with yeast showed that even highly resistant microorganisms suffer selective pressures against lytic peptide production. Thus, it

seemed unlikely that less resistant microorganisms would survive long enough to spread lytic peptides throughout an entire termite colony. To date, we have cultured over 25 different bacteria species from Formosan subterranean termites and surveyed their distribution in termite colonies from different geographical regions. Thus, there are many possible bacterial host species available as soon as natural tolerance against lytic peptides and transformation conditions are established.

Discussions with a panel of representatives from Dow AgroSciences covering technical development, commercial application, and legal issues connected to the potential release of genetically engineered microorganisms, indicated that pest control industry would favor a yeast-based expression system, because of the low cost of production and ease of application. As soon as a fusion gene is developed that incorporates additional safety features (e.g. a ligand to a protozoa surface receptor and a gut protease specific activation site) a yeast-based bait system will be developed and permission for contained semi-field testing will be sought.

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Appendix A

Background Data to Support Work

Table 1. Raw data used to generate the growth curve of *Pilibacter termitis*

Hours	Countable Plate	Colonies on Plate				Cell Concentration (Cells/ml)
	(10^{-X})	1	2	3	Avg	(# col*10*10*10^{X})
0	3	35	36	31	34	3,400,000
4	4	11	1	5	5.67	5,666,667
8	no data	N/A	N/A	N/A	N/A	N/A
12	5	103	116	89	103	1,026,666,667
20	5	153	186	191	177	1,766,666,667
24	6	188	147	149	161	16,133,333,333
32	6	131	126	162	140	13,966,666,667
48	5	289	307	257	284	2,843,333,333
72	5	148	130	139	139	1,390,000,000
96	4	365	415	423	401	401,000,000
120	no data	N/A	N/A	N/A	N/A	N/A
144	4	73	85	72	76.7	76,666,667
192	3	31	32	22	28.3	28,333,333

Table 2. Raw data used to generate the percentage mortality graph for the defaunation experiment where termites were fed with Metronidazole (2g/L)

	Day of count																																																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Treatment																																																	
Dish 1 (Metranidazole)	100	99	97	95	95	90	90	88	87	85	82	75	73	72	72	72	72	72	69	69	69	69	69	63	59	56	56	53	48	46	44	39	38	36	31	26	26	25	23	22	20	19	18	17	17
Dish 2 (Metranidazole)	100	100	100	100	100	86	86	84	84	81	81	75	75	75	75	74	74	73	68	68	68	68	66	60	59	57	57	54	53	51	49	46	45	42	38	34	27	26	24	23	22	19	19	19	19
Dish 3 (Metranidazole)	100	98	98	97	95	89	89	88	88	86	86	80	80	80	80	80	80	80	76	76	76	76	76	71	71	70	69	66	63	58	55	50	50	46	43	36	34	31	30	30	30	29	27	25	25
Dish 4 (Water)	100	100	100	100	100	95	95	94	94	93	92	87	87	87	86	86	85	85	82	82	82	82	82	78	74	71	70	67	66	63	62	57	56	53	47	43	36	31	30	28	28	28	28	28	28
Dish 5 (Water)	100	100	100	100	100	92	92	92	92	92	91	86	85	85	85	85	85	85	82	82	82	82	82	78	77	76	76	73	71	71	71	70	70	68	65	65	65	62	62	61	61	58	58	57	56
Dish 6 (Water)	100	100	99	99	99	93	93	92	92	92	92	87	87	87	87	87	87	87	84	83	83	83	83	77	74	72	71	68	68	67	65	65	64	64	61	59	55	54	54	52	52	52	52	50	46

Table 3. Raw data showing the statistical significance of *Kluyveromyces lactis* treatment effect on *Tetrahymena pyriformis* cultures in a pair-wise comparison with the control (*T. pyriformis* culture with no yeast present) at 72 hrs post exposure (SAS Proc Mixed ANOVA, Tukey's mean separation, 4 replications)

	Number of *Tetrahymena* at 72 hrs	p value	Standard Deviation (from experiment data points)
K. lactis -Vector	22.33	0.9911	4
K. lactis -MBP	11	< 0.0001	6
K. lactis HecGFP-1	9.8	< 0.0001	8
K. lactis HecGFP-8	18.66	0.0055	7
K. lactis HecGFP-6	17.16	0.0016	6
K. lactis Hec-1	9	< 0.0001	5
K. lactis Hec-2	19.66	0.0121	6
K. lactis Hec-3	19	0.0072	4
Control (no yeast present)	33.5		10
degrees of freedom	45		
standard error	3.6515		

Appendix B

Publications and Presentations

Bookchapter:

Husseneder, C, and Collier, R. E. 2008. Paratransgenesis for termite control. In: *Insect Symbiosis*. Bourtzis, K. and Miller, T.A. (eds.). CRC Press LLC, Boca Raton, Florida. Submitted.

Other publications

Husseneder, C., Collier, R.E. 2007. Paratransgenesis for termite control-constructing the enemy within. Proceedings of the International Congress of Insect Biotechnology & Industry, Daegu, Republic of Korea, Entomol Res 37: p A40.

Husseneder, C. 2007. Molecular genetic methods help unravel termite mysteries. Louisiana Agriculture 50: 16-17.

Husseneder, C., Collier, R.E., Wise, B.R. 2006. Paratransgenesis in termites. Proceedings of the 2006 National Conference on Urban Entomology, Raleigh, North Carolina: pp 144-146.

Invited Speaker

Husseneder, C., Collier, R. E., Colby, D. 2007. Genetic manipulation and its potential for control of termites and other urban pests. Annual Meeting of the Entomological Society of America, San Diego, California.

Husseneder, C., Collier, R.E., Foil, L.D. 2007. Paratransgenesis for termite control-constructing the enemy within. International Congress of Insect Biotechnology & Industry, Daegu, Republic of Korea.

Husseneder, C. and Collier, R. E. 2007. Paratransgenesis for termite control-constructing the enemy within. Seminar at North Carolina State University (Raleigh, North Carolina).

Husseneder, C. 2006. Paratransgenesis – research and development towards the future of pest control. Seminar at Dow AgroSciences (Indianapolis, Indiana).

Husseneder, C., Collier, R.E., and Wise, B.R. 2006. Paratransgenesis in termites. National Conference of Urban Entomology, Raleigh, North Carolina.

Submitted Oral Presentations

Collier, R.E., Husseneder, C., Foil, L. Cooper, R. and Enright F. 2006. Paratransgenesis – constructing the enemy within. Annual Meeting of the Entomological Society of America, Indianapolis, Indiana.

Husseneder, C., Foil, L. Cooper, R. and Enright F. 2006. Paratransgenesis for termite control. Biotechnology Committee, Baton Rouge, Louisiana.

Posters

Husseneder, C., Collier, R.E., Foil, L., Cooper, R. and Enright F. 2007. Paratransgenesis for termite control. Partners in Environmental Technology Technical Symposium & Workshop, Department of Defense, Washington, D.C.

Collier, R.E., Husseneder, C., Foil, L. Cooper, R. and Enright F. 2006. Paratransgenesis – constructing the enemy within. Insect Molecular Biology, Tucson, Arizona.

Collier, R.E., Husseneder, C., Foil, L. Cooper, R. and Enright F. 2006. Paratransgenesis – constructing the enemy within. Biotechnology Education of Teachers and Students, Baton Rouge, Louisiana.

Patent Applications

Provisional Application for Patent; May 17, 2007, for Paratransgenesis to Control Termites and Other Insects

Entomological Society of America Annual Meeting, 2006
Paratransgenesis: Constructing the enemy within
Wednesday, 13 December 2006 - 1:35 PM

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Paratransgenesis is the genetic manipulation of a host's symbiotic microorganisms to achieve an array of objectives, ranging from disease eradication to pest control. The application of paratransgenesis is promising in social insects, because social interactions promote the exchange of microbes naturally between individuals within colonies through mutual feeding and grooming. Within the social insects, termites are known for their close relationship with microbial symbionts. The hindgut of the Formosan subterranean termite (FST) workers provides a refuge for an array of protozoa and bacteria that fulfill important functions in the survival of their hosts, such as cellulose digestion. These symbionts are excellent tools and targets for paratransgenesis. In these studies aimed at the control of FST, the paratransgenesis targets are the termite's protozoa, which digest cellulose into metabolites subsequently utilized by the termite host. Without their protozoa and the nutrients they supply, termites die. Using microinjection, a technique developed during this study, defaunation of the termite gut has been observed with selected toxins within 72hrs of treatment. The genes of the successful toxins were synthesized and a proto-type gene-shuttle system was constructed to deliver toxins into the termite hindgut. The yeast, *Kluyveromyces lactis*, has been engineered to secrete toxins and is being used in termite feeding experiments to defaunate termites. It is the ultimate aim of this study to optimize the gene-shuttle for inducible expression within the recently characterized termite specific anaerobic, gram-positive bacterium, *Pilibacter termitis*, to minimize environmental impacts and achieve efficient colony level control of FST.

Appendix C

K. lactis Protein Expression Kit

Recombinant Protein Expression in Yeast

Instruction Manual

Catalog #E1000SStore at –20°C

Version 2.05/07

Table of Contents:

Supplied Kit Components:

The *K. lactis* Protein Expression Kit contains all reagents necessary to express a recombinant protein in the yeast *Kluyveromyces lactis*.

Vectors:

pKLAC1 Vector 20 µg
pKLAC1-*malE* Control Plasmid 20 µg

Restriction Enzyme:

Sac II 2,000 units
NEBuffer 4 (10X)

Integration Identification Primers:

Integration Primer 1 (10X)
5´ d(TACCGACGTATATCAAGCCCA) 3´ 500 µl

Integration Primer 2 (10X)
5´ d(ATCATCCTTGTCAGCGAAAGC) 3´ 1,000 µl

Integration Primer 3 (10X)
5´ d(CAGTGATTACATGCATATTGT) 3´ 500 µl

Yeast Transformation Reagents:

K. lactis GG799 Competent Cells 5 reactions
NEB Yeast Transformation Reagent 5 ml

Yeast Media:

Yeast Carbon Base (YCB) Medium Powder 12 grams
Acetamide Solution (100X) 10 ml

Method Overview:

The *K. lactis* Protein Expression Kit provides a method for cloning and expressing a gene of interest in the yeast *Kluyveromyces lactis* (Figures 1 and 2). Proteins may be expressed intracellularly or be secreted from the cell using the supplied expression vector pKLAC1 (Figure 3).

Secretion of proteins is the most common approach to *K. lactis* protein expression (1). To achieve protein secretion, the gene of interest is cloned into pKLAC1 downstream of the *K. lactis* a-mating factor domain (a-MF; Figure 4), resulting in expression of an a-MF fusion protein. The a-MF domain directs the fusion protein to be efficiently transported through the yeast secretory pathway. An a-MF fusion protein undergoes sequential processing by signal peptidase in the endoplasmic reticulum (ER) and the Kex protease in the Golgi, resulting in the secretion of a native form of the protein of interest into the growth medium (Figure 1).

Expression in yeast is driven by a mutant form of the strong *K. lactis LAC4* promoter ($P_{LAC4\text{-}PBI}$) that has been engineered to lack background *E. coli* transcriptional activity (2). Therefore, genes encoding products toxic to *E. coli* can be cloned into pKLAC1 in *E. coli* prior to their introduction into yeast cells. To achieve expression in yeast, pKLAC1 containing a cloned gene of interest is linearized by either Sac II or BstX I to produce an expression cassette that can integrate into the *K. lactis* genome at the *LAC4* locus by homologous recombination. A fungal acetamidase gene (*amdS*) in pKLAC1 provides for selection of yeast containing an integrated expression cassette by allowing their growth on nitrogen-free minimal medium containing acetamide. Only cells expressing *amdS* can break down acetamide to ammonia for use as a nitrogen source. An advantage of this selection method is that it enriches transformant populations for cells that have integrated multiple tandem copies of the expression cassette and therefore produce more recombinant protein. Finally, the supplied *K. lactis* GG799 strain is an industrial isolate that has no auxotrophies, rapidly grows to high cell density, and efficiently secretes heterologous proteins.

Secretion of both eukaryotic (2-7) and prokayotic proteins (8,9) from *K. lactis* has been achieved. Typically, proteins that are normally secreted from cells (e.g., cytokines, serum albumins, antibody fragments and glycosidases) produce the highest yields. In these cases, 10–50 mg of recombinant protein per liter can often be achieved in shake flasks, and yields can be further enhanced by high density cell fermentation. Secretion of other types of proteins is also possible. Secreted recombinant proteins are routinely detected in the growth medium of saturated cultures via SDS-PAGE and protein staining, Western analysis or enzyme assay. Secreted proteins may also bear post-translational modifications (e.g., asparagine-linked glycosylation) that can be removed by treatment with Endo H (NEB #P0702) or PNGase F (NEB #P0704).

Advantages:

- High level expression of recombinant proteins
- Rapid high cell density growth
- No background gene expression during *E. coli* cloning steps
- Easy and fast cell transformation procedure
- No expensive antibiotics required
- Attractive commercial sublicensing

Strategies for Protein Expression Using pKLAC1:

pKLAC1 can be used for either intracellular or secreted expression of proteins of interest in *K. lactis*. The following sections describe various factors that influence how genes should be cloned into pKLAC1 to achieve the desired method of expression.

Secreted protein expression using pKLAC1

Secretion of a protein of interest from *K. lactis* cells is the most common approach to protein expression. Secretion results in production of proteins that are significantly pure, that do not require difficult lysis of yeast cells to isolate, and that may have desired post-translational modifications (e.g. glycosylation) that cytosolic proteins do not. Protein secretion using pKLAC1 is achieved by generating a fusion between the protein of interest and the a-MF secretion domain present in the vector. To do this, a gene or open reading frame of interest must be inserted into pKLAC1 so that it is in the same translational reading frame as the a-MF domain. It is also required to have a Kex protease processing site (KR↓) at the junction between the a-MF domain and the N-terminus of the protein of interest.

Because fusion proteins will be processed by the Kex protease in the Golgi, there are two strategies for making fusions to the a-MF domain. The first strategy generates a secreted recombinant protein with a native N-terminus and the second produces a protein with additional vector-encoded amino acids at its N-terminus. The first strategy makes use of the unique XhoI site that lies immediately upstream of DNA encoding the Kex protease cleavage site. A gene cloned into XhoI must contain DNA at its 5´ end that reconstitutes the Kex protease cleavage site to allow for processing of the pro-protein (see Cloning Strategy I). In cases where the gene of interest contains an XhoI site or a native N-terminus is not required on the protein of interest, in-frame fusion with the a-MF domain may be achieved by cloning a gene into any of the other restriction sites that reside in the polylinker (see Cloning Strategy II). Fusion proteins made this way will contain additional amino acids encoded by vector DNA that resides between the Kex site and the polylinker restriction sites.

Native signal sequences

Naturally secreted proteins contain native secretion leader sequences. While various signal sequences can direct secretion of certain proteins from *K. lactis* (2–8), it is recommended to replace the protein's native leader sequence with the *K. lactis* a-MF domain sequence present in pKLAC1. Predictive algorithms such as SIGNAL P (http://www.cbs.dtu.dk/services/SignalP/) can be used to determine if a protein contains a native secretion leader sequence.

Intracellular protein expression using pKLAC1

Because yeast cells are difficult to lyse, secretion is the most common approach to expression in *K. lactis*. However, it is possible to use pKLAC1 to express a protein intracellularly. In this case, it is not necessary to create a fusion to the a-MF domain. A gene of interest should be cloned into pKLAC1 using the unique HindIII site immediately upstream of the a-MF domain and any of the polylinker sites. This places the gene of interest immediately downstream of the strong $P_{LAC4\text{-}PBI}$ promoter. In this case, it is important to ensure that the desired gene begins with a methionine codon to initiate translation. If the desired gene does not begin with a methionine codon, one should be added by PCR or site-directed mutagenesis.

It is important to note that when expressing a gene in this manner, idenfication of properly integrated strains or multiply integrated strains by PCR (see Optional Methods on pages 25 and 26) is not possible using Integration Primer 2. Integration Primer 2 anneals to a region of pKLAC1 DNA that lies between the HindIII site and the polylinker, and is removed during cloning for intracellular expression. Instead, a custom reverse primer can be designed that anneals to the 5´ end of the gene of interest (for more details see *K. lactis* Protein Expression Kit FAQ #2.5 at www.neb.com) and can be used in place of Integration Primer 2 in the Optional Methods. Additionally, the a-Mating Factor Sequencing Primer (NEB #S1275, Figure 4) cannot be used to sequence the 5´ end of genes cloned in this manner. The LAC4mt Promoter Sequencing Primer (NEB #S1274, Figure 4) should be used instead.

Epitope tagging

To allow for detection of secreted recombinant protein in the culture medium by western blotting, it may be desirable to create a fusion to a peptide epitope tag. Cloning Strategy III illustrates the use of PCR to incorporate DNA encoding a C-terminal hemagglutinin (HA) peptide epitope and a stop codon into the gene of interest. While this method could be used to add any antibody epitope tag to the C-terminus of the target protein, the HA antibody epitope tag has performed exceptionally well for detection and small scale purification of heterologous proteins secreted from *K. lactis* due to its lack of cross-reactivity with native host proteins during western blotting applications.

Tagging secreted proteins with C-terminal polyhistidine sequences has been met with mixed results. If C-terminal His-tagging is desired, it is recommended to add a stretch of at least 10 histidine residues to the C-terminus of the target protein to offset potential amino acid loss by the action of host carboxypeptidases. Alternatively, N-terminal his-tagging can be achieved by engineering a polyhistidine sequence between the Kex protease site and the N-terminus of the target protein.

Incorporation of stop codons

It is assumed that the gene of interest will include a stop codon (TAG, TAA or TGA) at its 3´ end. If it does not, one should be engineered into the cloning strategy. Stop codons may be incorporated into reverse PCR primers as shown in Cloning Strategies I, II and III.

Cloned Genes must be free of SacII or BstXI restriction sites

Vector pKLAC1 containing the gene of interest must be linearized with either SacII or BstXI to create an expression cassette that can be stably integrated into the *K. lactis* genome at the *LAC4* locus upon its introduction into *K. lactis*. Therefore, the gene of interest must be free of either SacII or BstXI sites. If the gene contains both restriction sites, then either all SacII sites or all BstXI sites must be removed using site-direct mutagenesis prior to transforming *K. lactis* cells.

Sequencing

Genes cloned into vector pKLAC1 should be sequenced prior to transformation of *K. lactis*. The positions of pKLAC1-specific sequencing primers available from New England Biolabs are shown in Figure 1. The sequence of vector pKLAC1 (Genbank #AY968582) is available at www.neb.com or by email request to info@neb.com.

Cloning Strategy I:

Secretion of a protein with native N-terminus

Shown are PCR primers (blue background) designed for amplification of an example gene of interest (yellow background) for cloning into pKLAC1. The Forward Primer must contain an XhoI site and a Kex protease cleavage site (boxed) immediately followed by the first codon of the gene's open reading frame. The Reverse Primer must include a restriction site for cloning into any of the polylinker sites of pKLAC1 (BglII shown).**XhoIForward Primer**gene of interest**Reverse Primer**After Kex processing, the protein of interest (green) containing a native N-terminus issecreted.**Kex**protein of interest**D-Y-S-L-I-V-G5´ NNN CTC GAG AAA AGA ATG ACC AAC GAT GCT TTT GTCK R M T N D A F VKex5´...ATG ACC AAC GAT GCT TTT GTC3´...TAC TGG TTG CTA CGA AAA CAGGAT TAC TCT CTT ATA GTC GGC...3´CTA ATG AGA GAA TAT CAG CCG...5´CTA ATG AGA GAA TAT CAG CCG ATT TCT AGA NNN 5´D Y S L I V G STOPα-MF-K-R-M-T-N-D-A-F-VBglII**

Cloning Strategy II:

Secretion of a protein with non-native N-terminus

In cases where the gene of interest contains an XhoI site, one may clone the gene in-frame with the a-MF domain using other sites in the pKLAC1 polylinker. This strategy results in vector-encoded amino acids being added to the protein's N-terminus. Shown are PCR primers (blue background) designed for amplification of an example gene of interest (yellow background) for cloning into the BglII and KpnI polylinker sites of pKLAC1.**BglIIForward PrimerReverse Primer**After Kex processing, the secreted form of the protein of interest (green) contains extra vector-encoded amino acids at its N-terminus (dashed underline).**Kex**protein of interest**D-Y-S-L-I-V-G5´ NNN AGA TCT ATG ACC AAC GAT GCT TTT GTCM T N D A F VCTA ATG AGA GAA TAT CAG CCG ATT CCA TGG NNN 5´D Y S L I V G STOPα-MF-K-R-E-A-E-A-R-R-A-R-S-M-T-N-D-A-F-VKpnI**gene of interest**5´...ATG ACC AAC GAT GCT TTT GTC3´...TAC TGG TTG CTA CGA AAA CAGGAT TAC TCT CTT ATA GTC GGC...3´CTA ATG AGA GAA TAT CAG CCG...5´**

Cloning Strategy III:

Secretion of a protein with a C-terminal hemagglutinin epitope tag

Shown are PCR primers (blue background) designed for amplification of an example gene of interest (yellow background) having a C-terminal antibody epitope tag (hemagglutinin epitope [HA] shown). The Forward Primer should be designed as shown in Cloning Strategies I or II (Strategy I shown). The Reverse Primer should contain DNA encoding an HA epitope tag, a stop codon, and a restriction site for cloning into the polylinker of pKLAC1 (BglII shown).**XhoIForward Primer**gene of interest**BglIIReverse Primer**After Kex processing, the protein of interest (green) containing a C-terminal HA-eptitope(underlined) is secreted.**Kex**protein of interest**D-Y-S-L-I-V-G-Y-P-Y-D-V-P-D-Y-AHA-epitopeHA-epitope5´ NNN CTC GAG AAA AGA ATG ACC AAC GAT GCT TTT GTCK R M T N D A F VKex5´...ATG ACC AAC GAT GCT TTT GTC3´...TAC TGG TTG CTA CGA AAA CAGGAT TAC TCT CTT ATA GTC GGC...3´CTA ATG AGA GAA TAT CAG CCG...5´CTA ATG AGA GAA TAT CAG CCG ATA GGT ATA CTA CAA GGT CTA ATA CGT ATT TCT AGA NNN 5´D Y S L I V G Y P Y D V P D Y A STOP**α-MF-K-R-M-T-N-D-A-F-V

Cloning a PCR Fragment into pKLAC1:

The procedure below is for cloning a fragment produced by PCR into pKLAC1. This example assumes that the PCR fragment contains a 5´ XhoI site, and has a stop codon followed by a BglII site incorporated into its 3´ end (see Cloning Strategy I).

1. Prepare a PCR fragment of the gene of interest as outlined in Cloning Strategy I on page 11.
2. Digest 0.5 µg of pKLAC1 DNA with 10 units of XhoI and 10 units of BglII in 20 µl of 1X NEBuffer 3 (supplied as a 10X stock) supplemented with 100 µg/ml BSA (supplied as a 100 mg/ml stock) at 37°C for 2 hours.
3. Digest 0.5 µg of the PCR fragment with 10 units of XhoI and 10 units of BglII in 20 µl of 1X NEBuffer 3 supplemented with 100 µg/ml BSA at 37°C for 2 hours.
4. Add an equal volume of phenol:chloroform (1:1, v/v) to the restriction digests, mix and remove the aqueous (top) phase to a fresh tube. Repeat using only chloroform.

Alternatively, the DNA fragments can be isolated using one of the many commercially available fragment purification kits. If a kit is used, skip to Step 8.

5. Add 10 µg glycogen or tRNA as carrier to both digests, then add a 1/10th volume 3M sodium acetate, mix and add an equal volume of 100% isopropanol. Incubate at room temperature for 10 minutes.
6. Microcentrifuge at 12,000 x *g* for 15 minutes. Pour off the supernatant and gently rinse the pellet with 70% ethanol. Allow the pellet to air-dry (~10 minutes).
7. Resuspend each sample in 25 µl of 10 mM Tris-HCl, 1 mM EDTA (pH 8.0).
8. Mix: 2 µl pKLAC1 digest (~40 ng)
   10 µl PCR fragment (insert digest) (~200 ng)
   2 µl 10X T4 DNA Ligase Buffer
   14 µl deionized water
   1 µl (~400 units) T4 DNA Ligase (NEB #M0202S)
9. Incubate at 16°C for 2 hours to overnight.

Completed ligation reactions can be stored frozen at –20°C indefinitely prior to transformation.

10. Transformation of frozen competent NEB 5-alpha F' I_q Competent *E. coli* (NEB #C2992) is recommended using 2–4 µl of the ligation reaction.

Any competent E. coli strain can be used. However, blue-white screening is not possible with pKLAC1.

11. Prepare miniprep DNA from several transformants. Digest each vector with an appropriate restriction endonuclease to determine the presence of a cloned insert.

Expression vectors may be stored frozen at –20°C indefinitely.

Example Protocol:

An example experiment is described to illustrate creation of a *K. lactis* strain that is capable of secreting a protein of interest. This is accomplished by linearizing pKLAC1 containing a cloned gene of interest, inserting the linearized expression vector into the *K. lactis* genome by integrative transformation and growing cells to achieve secretion of the desired protein into the growth medium. A control pKLAC1 vector containing the *E. coli* malE gene (pKLAC1-*malE*) that encodes the maltose binding protein (MBP) is supplied with the kit and can be processed in parallel using this protocol. Integration of pKLAC1-*malE* into the *K. lactis* genome routinely yields cells that secrete > 25 mg MBP per liter of culture.

Linearization of pKLAC1 for integrative transformation of *K. lactis*

pKLAC1 containing any desired gene must be linearized to allow it to insert into the *K. lactis* genome at the *LAC4* locus (Figure 5). This is accomplished by digesting the construct with either SacII (supplied with kit) or BstXI to generate an "expression cassette" consisting of > 6.2 kb of DNA containing $P_{LAC4\text{-}PBI}$, the cloned gene and the *amdS* selection cassette, and a 2.8 kb fragment containing the remaining pKLAC1 vector DNA. The cloned gene must be free of SacII sites (or BstXI sites if digesting with BstXI) to allow for generation of the proper expression fragment. It is not necessary to purify the expression fragment from the remaining vector DNA following digestion as only the expression fragment will integrate into the *K. lactis* genome upon transformation.

1. Digest 2 µg of pKLAC1 DNA containing the gene of interest with 20 units of SacII in 50 µl of 1X NEBuffer 4 (supplied as a 10X stock) at 37°C for 2 hours.

*The pKLAC1-malE control vector can be linearized only with SacII due to the presence of a BstXI site in the malE gene.*1 3´ P_{LAC4}gene of interestamdS5´ P_{LAC4}SacIISacII Linearized Expression Cassette3´ P_{LAC4}amdS5´ P_{LAC4}Genomic Insertion5´3´*LAC4*P_{LAC4}3´*LAC4*P_{LAC4}5´*K. lactis* chromosome3´P_{LAC4}5´*K. lactis* chromosomeIntegrated Expression CassetteamdSgene of interestgene of interestSacIISacIISacII

Figure 5: *Genomic integration of a linear expression cassette. Vector pKLAC1 containing the gene of interest is digested with either SacII or BstXI (SacII shown) and introduced into K. lactis cells. The 5´ P_{LAC4} and 3´ P_{LAC4} sequences direct insertion of the cassette into the promoter region of the LAC4 locus in the K. lactis genome.*

2. Desalt digested DNA using a commercially available DNA fragment purification kit (e.g., Qiagen's QIAquick™ PCR Purification Kit).

A total of 1 µg of linearized DNA in a volume less than 15 µl will be needed to transform K. lactis cells. DNA may be stored frozen at –20°C for up to one month prior to transforming K. lactis cells.

Transformation of *K. lactis* GG799 cells

Introduction of the linearized expression cassette into *K. lactis* cells is achieved by chemical transformation using the *K. lactis* GG799 Competent Cells and NEB Yeast Transformation Reagent supplied with the kit. This procedure yields approximately 1 x 10_4 transformants per microgram of DNA. Transformants are selected by growth on Yeast Carbon Base (YCB) Agar Medium containing 5 mM acetamide (see Media & Solutions). YCB medium contains glucose and all nutrients needed to sustain growth of *K. lactis* GG799 cells except a simple nitrogen source. Cells can utilize acetamide as a source of nitrogen only after it is broken down to ammonia by acetamidase, the product of the *amdS* gene present in pKLAC1.

The following steps should be conducted using aseptic technique. Care should be taken to ensure that pipet tips, tubes, solutions and deionized water are sterilized prior to use.

1. Thaw a tube of *K. lactis* GG799 Competent Cells on ice. Add 620 µl NEB Yeast Transformation Reagent to the cells. Briefly shake or invert the tube until the solution is homogeneous.

   Do not vortex.

2. Add 1 µg of linearized pKLAC1 DNA containing the gene of interest to the cell mixture. Briefly shake or invert the tube to mix.

   Do not vortex. The total volume of transforming DNA should not exceed 15 µl

3. Incubate the mixture at 30°C for 30 minutes.
4. Heat shock the cell mixture by incubation at 37°C for 1 hour in a water bath.
5. Pellet cells by microcentrifugation at ~7000 r.p.m for 2 minutes and discard the supernatant.
6. Resuspend the cell pellet in 1 ml sterile deionized water.
7. Pellet cells by microcentrifugation at ~7000 r.p.m for 2 minutes and discard the supernatant.
8. Resuspend the cell pellet in 1 ml YPGlu medium (see Media & Solutions) and transfer the cell mixture to a sterile culture tube. Incubate with shaking (250–300 r.p.m.) at 30°C for 30 minutes.
9. Transfer the cell mixture to a sterile 1.5 ml microcentrifuge tube. Pellet the cells by microcentrifugation at ~7000 r.p.m for 2 minutes and discard the supernatant. Resuspend the cell pellet in 1 ml sterile deionized water.

10. Remove 10, 50 and 100 µl of the cell suspension to separate fresh sterile 1.5 ml microcentrifuge tubes each containing 50 µl of sterile deionized water. Mix briefly and spread the entire cell mixture from each tube onto separate YCB Agar Medium plates containing 5 mM acetamide (see Media & Solutions). Incubate plates inverted at 30°C for 3–4 days until colonies form.

Due to the high transformation efficiency of K. lactis GG799 Competent Cells, plating multiple dilutions of the cell mixture is necessary to ensure formation of plates with distinct single colonies. Growth time should not exceed 5 days as small colonies that lack an integrated expression fragment may form. Plates containing colonies can be stored at 4°C for up to two weeks.

11. Streak or patch 10–20 individual colonies onto fresh YCB Agar Medium plates containing 5 mM acetamide. Incubate at 30°C for 1–2 days.

Patches of approximately 1.0 cm^2 are recommended. Plates containing patched cells may be stored at 4°C for up to 3 days prior to performing whole-cell PCR (optional steps 12, 13).

12. [OPTIONAL] Transformants can be tested to verify that they have correctly integrated the expression fragment (see Identification of Properly Integrated Cells, page 25).

13. [OPTIONAL] Correctly integrated transformants can be further screened to identify cells that have integrated multiple tandem copies of the expression fragment (see Identification of Multicopy Integrants, page 26).

Growth of strains for detection of secreted protein

1. From the patch of each strain that contains an integrated expression fragment, harvest cells from an area approximately 2 mm^2 by scraping with a sterile pipette tip and resuspend the cells in 2 ml of YPGal medium (see Media & Solutions) in a sterile culture tube. Incubate the cultures with shaking (~250 r.p.m.) at 30°C.

   The duration of growth will vary depending on the protein being secreted. As a general rule, allow a minimum of 2 days growth at 30°C to obtain a saturated culture (a culture density of > 30 OD_{600} units/ml). Analysis of culture supernatant may be performed each day thereafter to determine the optimum growth time to achieve maximum secretion of the protein of interest. Culture sizes will ultimately depend on the desired application. For example, to determine the efficiency of secretion on previously untested cells, 2 ml cultures allow for simultaneous analysis of many strains. Larger cultures (e.g., ≥1 L) for protein purification should be inoculated 1:100 with a starter culture grown overnight at 30°C.

2. Microcentrifuge 1 ml of each culture for 30 seconds to pellet cells. Remove the culture supernatant to a fresh microcentrifuge tube and store on ice

3. Since expression levels of recombinant proteins secreted from *K. lactis* vary from protein to protein, culture supernatant samples must be analyzed to determine if the protein of interest is being secreted. Polyacrylamide gel electrophoresis followed by Coomassie or silver staining of unconcentrated culture supernatant (15 µl per lane) allows for visual detection of proteins that are highly secreted (e.g. > 10 mg/l) (Figure 6A). Alternatively, Western blotting can detect lesser quantities of secreted protein (Figure 6B). If an antibody to your protein of interest is unavailable, an antibody epitope tag (e.g. hemagglutinin (HA) peptide epitope) can be engineered as a C-terminal fusion to the protein of interest (see Cloning a PCR Fragment, Strategy III). Finally, if the protein of interest is an enzyme, culture supernatant may be analyzed for the presence of the protein directly by activity assay (Figure 7). In such cases, it is important to note that the absence of an enzyme activity in culture supernatant does not always indicate a lack of secretion. For example, the enzyme may be secreted in an inactive form or the nutrient rich growth medium may inhibit the activity assay. In such cases, absence of secretion of the protein of interest should also be confirmed by SDS-PAGE or Western analysis.

4. *K. lactis* cells can be stored at –70°C suspended in a final concentration of 20% (v/v) sterile glycerol. For example, 500 µl of a culture of freshly grown cells can be diluted with 500 µl of a sterile 40% glycerol solution (to give a 20% final glycerol concentration). Alternatively, a scoop of cells from a freshly grown streak on agar medium can be scraped from the plate using a sterile loop or pipet tip and resuspended directly in 20% sterile glycerol. It is improtant to make sure that all solutions and tubes that contact the cells are sterilized prior to their use. To revive frozen *K. lactis* cells containing an integrated pKLAC1 construct, streaka small aliquot of frozen cells on YCB agar medium supplemented with 5 mM acetamide. After this initial growth on YCB agar medium, the cells can be grown without selection in rich medium for protein expression.

Optional Method: Identification of Properly Integrated Cells

Transformants in which the expression cassette has correctly integrated into the *K. lactis* genome can be identified by PCR using supplied Integration Primers 1 and 2 to amplify a 1.9 kb product (Figure 8A). To facilitate simultaneous screening of many transformants, PCR using freshly grown cells as a source of template chromosomal DNA is recommended.

1. For each transformant patched and grown on YCB Agar Medium plates containing 5 mM acetamide (see Example Experiment step 1), harvest cells from an area approximately 1 mm^2 by scraping with a pipette tip and resuspend the cells in 25 µl of 1 M sorbitol containing 2 mg/ml Lyticase (Sigma #L-2524). Mix by vortexing. Incubate at 30°C for 1 hour.

2. Lyse the Lyticase-treated cells in a thermocycler at 96°C for 10 minutes.

3. 10 µl 10X Integration Primer 1
   10 µl 10X Integration Primer 2
   10 µl 2 mM dNTPs (NEB #N0447, 10 mM stock)
   10 µl 10X ThermoPol Buffer (NEB #B9004)
   1 µl Taq DNA Polymerase (NEB #M0267 or NEB #M0273)
   34 µl deionized water
   100 µl final reaction volume

4. Thermocycling should consist of 30 rounds (95°C for 30 seconds, 50°C for 30 seconds and 72°C for 2 minutes), followed by incubation at 72°C for 10 minutes.

5. Analyze 10 µl of each amplification reaction on a 1% agarose gel.

Integration of the expression fragment at the LAC4 locus in the K. lactis genome will result in amplification of a 1.9 kb product (Figure 8A).

6. Test strains harboring a properly integrated expression fragment for secretion of the protein of interest (see Growth of strains for detection of secreted protein, page 21).2

Optional Method: Identification of Multi-copy Integrants

It is possible for up to 10 copies of the expression cassette to tandemly insert into the genome during transformation. Strains harboring multiple integrations often produce more secreted protein. An advantage of selection for *K. lactis* transformants on YCB Agar Medium containing acetamide is that it enriches for cells harboring multiple tandem integrations (Figure 8B). Multiply integrated cells can be identified using whole-cell PCR with Integration Primers 2 and 3.

1. For each transformant patched and grown on YCB Agar Medium plates containing 5 mM acetamide (see Example Experiment step 1), harvest the cells from an area approximately 1 mm^2 by scraping with a pipette tip and resuspending in 25 µl of 1 M sorbitol containing 2 mg/ml Lyticase (Sigma #L-2524). Mix by vortexing. Incubate at 37°C for 1 hour.

2. Lyse the Lyticase-treated cells in a thermocycler at 96°C for 10 minutes.

3. 10 µl 10X Integration Primer 2
   10 µl 10X Integration Primer 3
   10 µl 2 mM dNTPs (NEB #N0447, 10 mM stock)
   10 µl 10X ThermoPol Buffer (NEB #B9004)
   1 µl Taq DNA Polymerase (NEB #M0267 or NEB #M0273)
   34 µl deionized water
   100 µl final reaction volume

4. Thermocycling should consist of 30 rounds (95°C for 30 seconds, 50°C for 30 seconds and 72°C for 3 minutes), followed by incubation at 72°C for 10 minutes.

5. Analyze 10 µl of each amplification reaction on a 1% agarose gel.

Cells harboring multiple tandem integrations of the expression fragment at the LAC4 locus in the K. lactis genome will result in amplification of a 2.3 kb product (Figure 8B).

6. Test strains harboring multiple copies of the expression fragment for secretion of the protein of interest (see Growth of strains for detection of secreted protein, page 21).

Media & Solutions

40% Glucose or 40% Galactose Stock Solutions (1 liter)

Add 400 g glucose or galactose to 500 ml deionized water

Dissolve by stirring, warm to 50°C to aid dissolution if needed.

Adjust volume to 1 liter with deionized water; dispense into 100 ml aliquots

Filter sterilize or autoclave for 20 minutes at 121°C.

Solutions may carmelize if autoclaved longer or at temperatures higher than recommended.

YPGlu & YPGal Media (1 liter)

Dissolve 10 g Yeast Extract and 20 g Bacto™ Peptone (Becton Dickinson #211820) in 950 ml deionized water

Autoclave for 20 min at 121°C; let cool to room temperature

Aseptically add 50 ml of sterile 40% glucose (for YPGlu) or 40% galactose (for YPGal)

Solid YPGlu and YPGal media can be made by adding 20 grams of Bacto™ agar (Becton Dickinson #214050) to the recipe prior to autoclaving.

1 M Tris-HCl Buffer Stock Solution pH 7.0 (1 liter)

Dissolve 121.14g Tris (American Bioanalytical #AB14042) in 800 µl deionized water. Adjust pH to 7.0 with the appropriate volume of concentrated HCl. Bring final volume to 1 liter with deionized water.

Autoclave. Store at room temperature.2

YCB Agar Medium with 5 mM acetamide (500 ml)

Mix in an autoclavable bottle:

15 ml 1 M Tris-HCl Buffer Stock Solution

5.85 g YCB Medium powder (supplied with kit)

10 g Bacto agar (Becton Dickinson #214050)

Bring volume up to 495 ml with deionized water

Autoclave 20 minutes at 121°C. Let cool to ~60°C.

Aseptically add:

5 ml 100X Acetamide Stock Solution (supplied with kit)

Dispense into sterile disposable Petri dishes; close plates and let sit at room temperature until solid, then invert and let sit for 12–18 hours to dry prior to use.

Yeast carbon base (YCB) medium contains glucose and all nutrients needed to sustain growth of K. lactis GG799 Competent Cells except a simple nitrogen source. Cells can utilize acetamide as a source of nitrogen only after it is broken down to ammonia by acetamidase (the product of the amdS gene present in pKLAC1). ***Acetamide should not be autoclaved.***0

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